CANCER RESEARCH

VOLUME 7
NUMBER 3
MARCH, 1947

A MONTHLY JOURNAL
OF ARTICLES AND ABSTRACTS
REPORTING CANCER RESEARCH

CONTENTS

THE OFFICIAL ORGAN OF THE
AMERICAN ASSOCIATION FOR CANCER RESEARCH, INC.

Fourth International Cancer Research Congress,
St. Louis, Missouri, September 2-7, 1947.

CANCER RESEARCH

This journal is sponsored by the American Association for Cancer Research, Inc.; The Anna Fuller Fund; The Donner Foundation, Incoporated; Cancer Research Division; The Jane Coffin Childs Memorial Fund for Medical Research, and the Elsa U. Pardee Foundation.

Published by The Donner Foundation, Incorporated, Cancer Research Division.
Publication Office, 125 Temple Street, New Haven 10, Connecticut

The annual subscription rates for one volume are: To members of the American Association for Cancer Research, Inc., $5.00; to others and to libraries, institutions, and organizations, $7.00. Business communications, remittances, and subscriptions should be addressed to Theodore P. Eberhard, Business Manager, 2136 Land Title Building, Philadelphia 10, Pa.

Entered as second class matter December 16, 1946, at the Post Office at New Haven, Conn., under the Act of March 3, 1879.

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On the Sensitivity of Different Tissues in Street Strain Mice to 9,10-Dimethyl-1,2-Benzanthracene

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(Received for publication October 18, 1946)

It is apparent from recent investigations into the action of carcinogenic hydrocarbons on mice of inbred strains that new growths may be produced in other organs than the site of application. This holds, however, only for the kinds of tumor which occur spontaneously in the strain in question, these tumors appearing earlier and/or in increased numbers. Simultaneously in 1939 Brues and Marble (3), Engelbreth-Holm (4), and Mider and Morton (17) were the first to call attention to this acceleration of the spontaneous development of tumors. Following the application of carcinogenic hydrocarbons they observed an acceleration of the development of leukemia in the strains used. These investigations have later been confirmed and elaborated by the same researchers as well as others: Morton and Mider (18), Law (11), Kirschbaum, Strong and Gardner (10), Kirschbaum and Strong (9), Kirschbaum and Kaplan (8), Engelbreth-Holm and Lefèvre (6), Engelbreth-Holm and Poulsen (7), and Lefèvre (12).

Acceleration has also been observed of mammary carcinomas occurring in certain strains of mice by Maisin and Coolen (14), Engelbreth-Holm (5), Engelbreth-Holm and Lefèvre (6), Engelbreth-Holm and Poulsen (7), Lefèvre (12) and of pulmonary adenomas by Andervont (1, 2), Lynch (13), and Lefèvre (12).

The most important feature about this process of acceleration is the fact that only tumors encountered spontaneously lend themselves to acceleration. This fact forms the basis of Lefèvre's view (12) that carcinogenic hydrocarbons are only capable of "inducing" new growths in tissues where spontaneous tumors are met with, but not in others.

For the purpose of elucidating this question and, if possible, arriving at an explanation of the mechanism of the acceleration the writers of this paper started a series of experiments. One of these will be reported below.

In this series a certain carcinogenic hydrocarbon was applied in small doses directly to various tissues—tissues which develop tumors spontaneously as well as those which do not. The purpose of this procedure was to investigate whether the process of acceleration observed, *e.g.* following painting of the skin, is due simply to the fact that certain tissues are more susceptible to carcinogenic action (because of special inherited qualities) than others, and whether new growths can be produced in tissues which do not exhibit spontaneous malignant growths.

MATERIAL AND TECHNIC

The experiments were carried out on mice of the Street strain, aged 4 to 6 weeks. This strain has not been inbred by brother to sister mating for a sufficient number of generations to secure the complete genetic uniformity obtainable by a longer period of inbreeding. Genetically it is, however, so pure that the incidence of new growths in mice of this strain has been constant during latter years. Leukemia has been observed in about 1 to 2 per cent, mammary carcinoma in about 7 and 35 per cent in non-breeding and breeding mice respectively, and pulmonary tumors in about 3 per cent (12). Moreover, a few cases of tumors have been observed in other organs (carcinoma of the liver, hemangioma of the spleen, granulosa-cell tumor of the

* This investigation has been supported by grants from Anders Hasselbalchs Fond, Kong Christian den Tiendes Fond, Carl Scheplers og Hustrus Fond and The Danish Anti-Cancer League.

ovary, carcinoma of the small intestine, squamous-cell carcinoma of the skin).

The mice used for each experiment were litter mates, as far as possible an equal number of males and females. Half of each litter was left untreated, serving as controls.

In the experimental animals 0.02 mgm. of 9, 10-dimethyl-1, 2-benzanthracene dissolved in 0.01 cc. of paraffin was injected directly into one of the following tissues: thymus, lymph node, subcutaneous tissue, mammary gland, testis, lung, spleen, kidney, bone marrow.

The following technic was employed in injecting the hydrocarbon: Injection into the thymus was made by plunging the needle from the upper border of the manubrium sterni, about 2 to 3 mm. down, immediately behind the sternum, where the hydrocarbon was placed. Before injecting an inguinal lymph node the skin was cut about 1 cm. medial to the lymph node. The skin was pulled to one side and injection made directly into the lymph node. The site of injection into the subcutaneous tissue was in the left flank, approximately midway between the axilla and groin. Injection into the mammary tissue was made by placing the hydrocarbon subcutaneously below one of the lower nipples. Injection was made into the testis after the latter had been made to descend into the scrotum by means of light pressure. Injection into the lung was made by pushing the needle through the abdominal wall immediately below the right costal border, through the diaphragm and up through approximately two-thirds of the length of the thorax. Before injecting the spleen the skin was incised longitudinally on the abdomen, about 1 cm. lateral to the midline; the spleen was grasped with a forceps through the abdominal wall. This maneuver fixed the position of the spleen sufficiently for making the injection through the abdominal musculature. The needle was plunged from the lower pole of the spleen through about two-thirds of its length. Injection into the kidney was performed with the same technic with the only difference that the kidney was exposed by pushing the overlying organs to one side by a gentle pressure with a forceps. The needle was plunged through the lower pole of the kidney upwards through about two-thirds of its length. Injection into bone marrow was made by injecting the hydrocarbon into the medullary canal of the femur. For this end the knee was placed in extreme flexion, the bone between the two condyles pierced, and the needle plunged into the medullary canal of the femur.

EXPERIMENTS

Table I shows the number of mice treated in these experiments, the number of mice reaching an age greater than 3, 6, 9, and 12 months respectively, and the number of survivors. Fifty to 100 mice were used in each experiment and a varying number of mice (up to one-fourth) still survive 13 to 15 months after the start of the experiments.

The death of a comparatively large number of mice within the first months after the injections can hardly be ascribed to the treatment. The mortality was mainly due to an epidemic of enteritis which prevailed among the mice for some length of time. This appears also from the fact that the mortality was, practically speaking, the same among experimental and control mice (Table I).

TABLE I

0.02 mgm. of 9,10-Dimethyl-1,2-benzanthracene injected into	No. of exper. mice: At start of exper. Aged 1 mo.	> 3 mos.	> 6 mos.	> 9 mos.	> 12 mos.	Surviving	No. of control mice: At start of exper. Aged 1 mo.	> 3 mos.	> 6 mos.	> 9 mos.	> 12 mos.	Surviving
Thymus	103	68	29	25	13	12	103	78	44	33	17	17
Inguinal lymph node	64	63	27	24	24	23	68	53	31	29	27	25
Subcutaneous tissue	98	68	44	18	17	15	96	68	41	23	17	16
Mammary gland	101	62	29	17	13	12	99	72	39	30	17	15
Testis	96	63	32	23	14	13	102	69	37	25	19	14
Lung	106	82	44	36	33	29	101	76	40	27	23	21
Spleen	80	61	39	29	27	20	79	66	36	20	16	14
Kidney	79	64	29	17	9	6	79	59	33	30	27	26
Bone marrow	50	44	22	21	20	20	49	48	24	24	21	21

Table II contains a survey of the tumors which so far have been observed among the experimental and control mice. In the fractions the numerator represents the number of tumors observed, and the denominator the number of survivors at the time of death of the first tumor-bearing mouse within the experiment in question. The adjacent columns give the survival time (in months) of the tumor-bearing mice.

TABLE II

0.02 mgm. of 9, 10-Dimethyl-1,2-benzanthracene injected into	No. of exper. mice exhibiting local tumors	Survival time, mos.	No. of exper. mice exhibiting distant tumors (thymus)	Survival time, mos.	No. of controls exhibiting tumors	Survival time, mos.
Thymus	8/68	3, 3, 3, 4, 4, 5, 7, 8	—		—	
Inguinal lymph node	—		2/54	4, 5	—	
Subcutaneous tissue	—		—		—	
Mammary gland	—		—		2/30	9, 11
Testis	—		—		—	
Lung	1/33	12	2/52*	5, 7	—	
Spleen	—		—		—	
Kidney	—		1/19	7	—	
Bone marrow	—		—		—	

* See Discussion.

Experimental as well as control mice exhibited only those kinds of tumor which are characteristic of the Street strain, *i.e.* leukemia, mammary carcinoma, and pulmonary tumor.

So far the controls have developed only 2 cases of spontaneous tumors, both mammary carcinomas. The

absence of leukemia and lung tumors is no doubt due to the relatively brief lapse of time since the experiments were performed, *i.e.* 13 to 15 months. It appears from earlier statistics of spontaneous tumors in the Street strain (12) that mammary carcinoma was observed in non-breeding animals from the age of 12 months and in breeding mice from the age of 6 months. Leukemia, on the other hand, usually did not appear until the age of 14 to 15 months, although a few cases were observed at the age of 7 to 8 months and one in a mouse aged only 4 months. Pulmonary tumors were not met with until the age of 19 months. Hence, the absence of leukemia and pulmonary tumor among the controls of the present experiment is in accordance with earlier observations.

Another feature apparent from the same statistics of spontaneous tumors in the Street strain (12) is that leukemia was usually generalized, exhibiting more or less marked leukemic changes in lymph nodes, spleen, and liver, and most often also lymphosarcomatous enlargement of the thymus. Only the case just mentioned of leukemia encountered in a 4 months old mouse, appeared in the form of a tumor of the thymus without leukemic changes in other organs.

As apparent from Table II injection of 0.02 mgm. of 9, 10-dimethyl-1, 2-benzanthracene into the thymus of 68 mice was followed by leukemic thymic tumors in 8 cases. The age of these 8 animals varied from 3 to 8 months. The same dose of hydrocarbon injected into an inguinal lymph node resulted in 2 cases of thymic tumor at the age of 4 and 5 months respectively among 54 mice. Following injection of the hydrocarbon into the lung, 2 cases of thymic tumor were observed at the ages of 5 and 7 months among 52 mice. In addition, a pulmonary adenoma was observed in a 12 months old mouse among a number of 33. Injection into the kidney was followed by 1 thymic tumor in a mouse aged 7 months.

Injection of the hydrocarbon into the subcutaneous tissue, mammary gland, testis, spleen, and bone marrow has so far failed to cause any tumors.

In all cases microscopical examination was made of the thoracic organs, the liver and kidney. The lymph nodes were examined in only a few cases, apart from the experiments in which injection was made into the lymph nodes.

Microscopical examination of the thymic tumors encountered revealed a picture exactly like the one met with in spontaneous leukemia. Nearly all cases exhibited invasive growth into the heart and pulmonary tissue. Moreover, nearly all the mice affected with thymic tumor had perivascular and peribronchial infiltrates in the central area of the lungs. Liver, kidney, and spleen failed to exhibit leukemic changes in most of the animals. Of the 8 mice dying from thymic tumor following injection into the thymus, only 1 exhibited a moderate leukemic infiltration in the liver. Moreover, 2 additional mice in the same experiment revealed leukemic changes in the kidneys. The lymph nodes were found to be of normal size except in one of the 2 cases exhibiting thymic tumor following injection into an inguinal lymph node. In addition to the thymic tumor, this mouse revealed some perivascular and diffuse infiltration in the liver and beginning leukemic changes in the peripheral lymph nodes which were slightly enlarged. These changes were rather more marked in the right inguinal lymph node which was the site of the injection, than in the other lymph nodes, but it was not a case of local lymphosarcoma. The other mouse, which had received the injection into an inguinal lymph node, failed to exhibit a similar condition. All its peripheral lymph nodes were normal.

Neither gross nor microscopical changes could be observed at the site of injection in those animals that died from thymic tumor following injection of the hydrocarbon into the lung or kidney.

The pulmonary adenoma met with in one mouse, aged 12 months and not affected with thymic tumor, proved to be a typical solitary adenoma, about 1 mm. in diameter. Still, pulmonary adenomas may have occurred in other instances, as microscopical examination was made only in cases with grossly visible changes. Therefore, adenomas visible by microscopical examination only, may have been overlooked.

During the 13 to 15 months' experimental period none of the experimental animals exhibited mammary carcinoma.

DISCUSSION

It appears from the experiments described above that injection of 0.02 mgm. of 9, 10-dimethyl-1, 2-benzanthracene in paraffin into mice of Street strain was followed by the development of leukemic tumors of the thymus only, apart from a single case of pulmonary adenoma.

Among the total of 13 thymic tumors 8 occurred following injection of the hydrocarbon into the thymus gland. Probably the 2 cases encountered following injection of the hydrocarbon into the lung belong to this experimental group, as the hydrocarbon may have been placed in a site whence it has been capable of acting directly upon the thymic tissue. Following injection into the lung the hydrocarbon in the paraffin solution usually settles in the pleural cavity between the pulmonary lobes. In a few cases of deaths within a couple of months of the injection, the paraffin was found in the pleural cavity immediately adjacent to the mediastinum.

One case of thymic tumor, occurring after injection of the hydrocarbon into the kidney, developed so late

(in a 7 months old mouse) that it may have been spontaneous, considering that spontaneous leukemic tumors of the thymus in Street mice are observed in animals aged 7 to 8 months (12). The same may apply to one of the thymic tumors encountered following injection into the lung (in a mouse aged 7 months) and to 2 of the 8 cases (in mice aged 7 and 8 months) following injection into the thymus. On the other hand, it is extremely unlikely that the remaining 6 thymic tumors, occurring in 3 to 5 months old mice among this group of 68, should be spontaneous. The same applies to the two cases occurring in mice aged 4 and 5 months respectively following injection of the hydrocarbon into a lymph node among a group of 54 animals. On the whole it must be kept in mind that the controls exhibited no case of leukemia.

The experiments have shown that 0.02 mgm. of 9, 10-dimethyl-1, 2-benzanthracene is capable of producing new growth in thymus only. In other words the thymus is considerably more susceptible to carcinogenic action than the other tissues investigated.

This accords well with the observation reported by McEndy, Boon and Furth (16). They found the incidence of spontaneous leukemia in mice of the high leukemia stock Ak to fall after thymectomy, from 77 to 8 per cent in females and from 61 to 11 per cent in males. The interpretation of these results by the authors is that the effect of thymectomy is due either to "removal of site of potentially malignant lymphoid cells" or "inhibition of neoplastic growth in general" or possibly both. The experiments described in the present paper appear to lend support to the theory that the thymus is the site of "potentially malignant cells."

Injection of the hydrocarbon into a lymph node, performed in one of the experiments, seems to indicate that the lymphatic tissue also is more susceptible to the action of carcinogenic hydrocarbon than the other organs investigated, excepting the thymus. As yet, however, the figures are too small to form the basis of definite conclusions.

As stated above, spontaneous leukemia in Street mice is usually attended with major or minor generalized leukemic tissue changes, frequently also with lymphosarcomatous degeneration of the thymus. In the present experiments, on the other hand, the leukemia was characterized by the development of a thymic tumor, in nearly all cases without leukemic changes in other organs.

It has been shown experimentally that leukemic changes develop in the course of a brief space of time, before the leukemia becomes manifest, in cases of spontaneous leukemia (Saxton, Boon and Furth [19]) as well as of induced leukemia (McEndy, Boon and Furth [15]). Considering the usually more rapid course of induced leukemia on the whole, it is not at all unlikely that the development of the thymic tumors occurring in the experiments described above was so quick that death ensued before major changes had time to manifest themselves. One cannot, however, rule out the possibility that the induced thymic tumors have had a stronger tendency to lymphosarcomatous growth without dissemination of the malignant cells than usually encountered in cases of spontaneous thymic tumors.

Injection of 0.02 mgm. of the hydrocarbon into the mammary tissue failed to result in the development of mammary carcinoma. One might be entitled to expect it, considering that painting with carcinogenic hydrocarbons (Engelbreth-Holm [4, 5], Engelbreth-Holm and Lefèvre [6], Lefèvre [12]) proved capable of producing an acceleration of spontaneous mammary carcinoma. In these cases it was, however, a question of experiments with other strains of mice, *i.e.* Aka and Little's dilute brown. Using Street mice Lefèvre (12) did not succeed in producing any acceleration of the development of mammary carcinoma by painting with 9, 10-dimethyl-1, 2-benzanthracene or with methylcholanthrene. Still, Engelbreth-Holm and Poulsen (7) have observed acceleration of mammary carcinomas in Street mice following ingestion of 9, 10-dimethyl-1, 2-benzanthracene. No explanation has as yet been found of this apparent disagreement. Thus, there seems to be a difference between the various strains exhibiting spontaneous mammary carcinoma as regards the acceleration of mammary growths. This difference is presumably of genetic origin or possibly due to a difference regarding the presence of the milk factor.

Lung tumors were not encountered in the present experiments, a surprising finding in view of the fact that Lefèvre (12), painting Street mice, found a marked acceleration of the development of lung tumors. Possibly the explanation is that the hydrocarbon has been used in too small doses or that the time of observation has been too short.

The experiments have clearly shown the *different susceptibility to a given dose of a given hydrocarbon on the part of the different* tissues. In the strain investigated, in which leukemia is the growth most easily accelerated, the thymus has proved to be far more susceptible than any other injected tissue, reacting by developing growths following action from 0.02 mgm. of hydrocarbon. This dose was unable to produce local growths in any other tissue in the course of the experimental period (about 1 year).

The tumors most commonly met with in the strain: mammary carcinoma and pulmonary adenoma, failed to appear within the period of observation following local injection of 0.02 mgm. of the hydrocarbon.

The great susceptibility of the thymus might indicate a reason for the accelerated development of leukemia following painting of the skin. Possibly, small quantities of hydrocarbon, absorbed from the skin and circulating in the organism, are sufficient to produce neoplastic growth in particularly susceptible tissues, but not in others.

SUMMARY AND CONCLUSION

9, 10-Dimethyl-1, 2-benzanthracene, 0.02 mgm., was injected into each of the following tissues: thymus, lymph node, subcutaneous tissue, mammary gland, testis, lung, spleen, kidney, bone marrow. Leukemia (lymphosarcomatous tumor of the thymus, in nearly all cases without other leukemic changes) was observed following injections, into the thymus, in 8 of 68 mice; into a lymph node, in 2 of 54 mice; into the lung, in 2 of 52 mice; and into the kidney, in 1 of 19 mice. Apart from a single pulmonary adenoma, no other growths were encountered. Injection of the hydrocarbon into the other organs mentioned failed to cause local tumor formation. The controls exhibited two cases of mammary carcinoma but no leukemia.

It is concluded that the thymus is more susceptible to the direct application of the hydrocarbon used than are the other tissues investigated.

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The Induction of Neoplasms in Five Strains of Rats with Acetylaminofluorene

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(Received for publication October 10, 1946)

The variety of tumors observed in rats fed with acetylaminofluorene suggests constitutional differences in response to this chemical. Wilson, DeEd and Cox (8), reported 29 carcinomas and 1 sarcoma in 39 rats of an inbred albino stock which had been fed 2-acetylaminofluorene in various concentrations. The most frequent sites were bladder and mammary gland. Bielschowsky (1) observed 105 neoplasms in 93 Wistar rats which ingested 4 mgm. daily for 30 weeks. The majority of neoplasms were malignant hepatomas, mammary cancer and cancer of the external acoustic duct. He observed no tumors of the urinary bladder, and found 23 of the 27 mammary cancers in intact females and the majority of malignant hepatomas to be in male rats. Aside from sex as a differential in the localization of these tumors, Bielschowsky (2) in a second paper reported the induction of multiple adenomas of the thyroid and 1 macroscopic adenocarcinoma of the thyroid in 10 rats fed 2-acetylaminofluorene in combination with allylthiourea. Lopez (6) reported a glioma as well as hepatoma and mammary tumors in rats fed this chemical. Heiman (5) reported 4 salivary gland and 2 parathyroid tumors in Wistar rats following the introduction of the acetylaminofluorene directly into the pharynx with a syringe and curved needle.

MATERIAL AND METHODS

The present experiment included 10 female rats from each of 5 different inbred lines. When the rats were 3 to 4 months of age they were placed on Sherman's mash (66 per cent whole wheat flour, 33 per cent whole milk powder and 1 per cent salt) as a control diet for 3 weeks. Then the acetylaminofluorene was added to the diet in a 0.05 per cent concentration. The mash containing the chemical was fed for 365 days and the experimental rats alive at the end of this period were continued on the control diet without the chemical for the remainder of their lives. The mash was so finely ground that it was impossible for the rats to select against the chemical but they ingested somewhat less than was fed to them, due to some unrecovered waste.

Table I shows that the rats of the several strains varied in their appetite for the control diet as well as in the amount of food they ingested after the chemical was added. The average consumption of the control diet varied from 8 gm. per rat per day for the Marshalls to 9.5 gm. for rats of the August strain. After the chemical was added to the diet the appetite of the rats diminished and the average for the experimental period varied from 6.1 gm. per rat per day for the Copenhagens to 7.2 gm. per rat per day for the Augusts. The amount of acetylaminofluorene fed varied from 3 mgm. per rat per day for rats of the Copenhagen strain to 3.6 mgm. per day for rats of the August strain. The average for the entire group was 3.4 mgm. per rat per day but as previously mentioned somewhat less than this amount was ingested, due to a certain amount of unrecoverable waste. At the end of the experimental feeding period, 7 of the Marshall, 5 of the August, 4 each of the Fischer and A × C rats had died and all of the Copenhagen rats were still living. The latter had been fed 10.6 gm. of acetylaminofluorene or an average of about 1 gm. per rat and the amount of chemical fed to the others varied from 5.7 gm. for the Marshalls to 10.9 gm. for the A × C rats.

TABLE I: FOR EACH STRAIN THE NUMBER OF RATS, AVERAGE DAILY CONSUMPTION OF CONTROL AND EXPERIMENTAL DIET, TOTAL AND AVERAGE DAILY AMOUNT OF ACETYLAMINOFLUORENE FED

			0.05% Acetylaminofluorene		
Strain	No. of rats	Control diet, gm./day	Average diet, gm./day	Total gm. Chem.	Average mgm. Chem. day
Marshall	10	8.0	6.9	5.7	3.4
August	10	9.5	7.2	10.1	3.6
Fischer	10	8.2	6.4	9.0	3.2
Copenhagen	10	8.2	6.1	10.6	3.0
A × C	10	9.4	7.1	10.9	3.5
Total	50	8.6	6.7	46.4	3.4
Control	10	7.8			

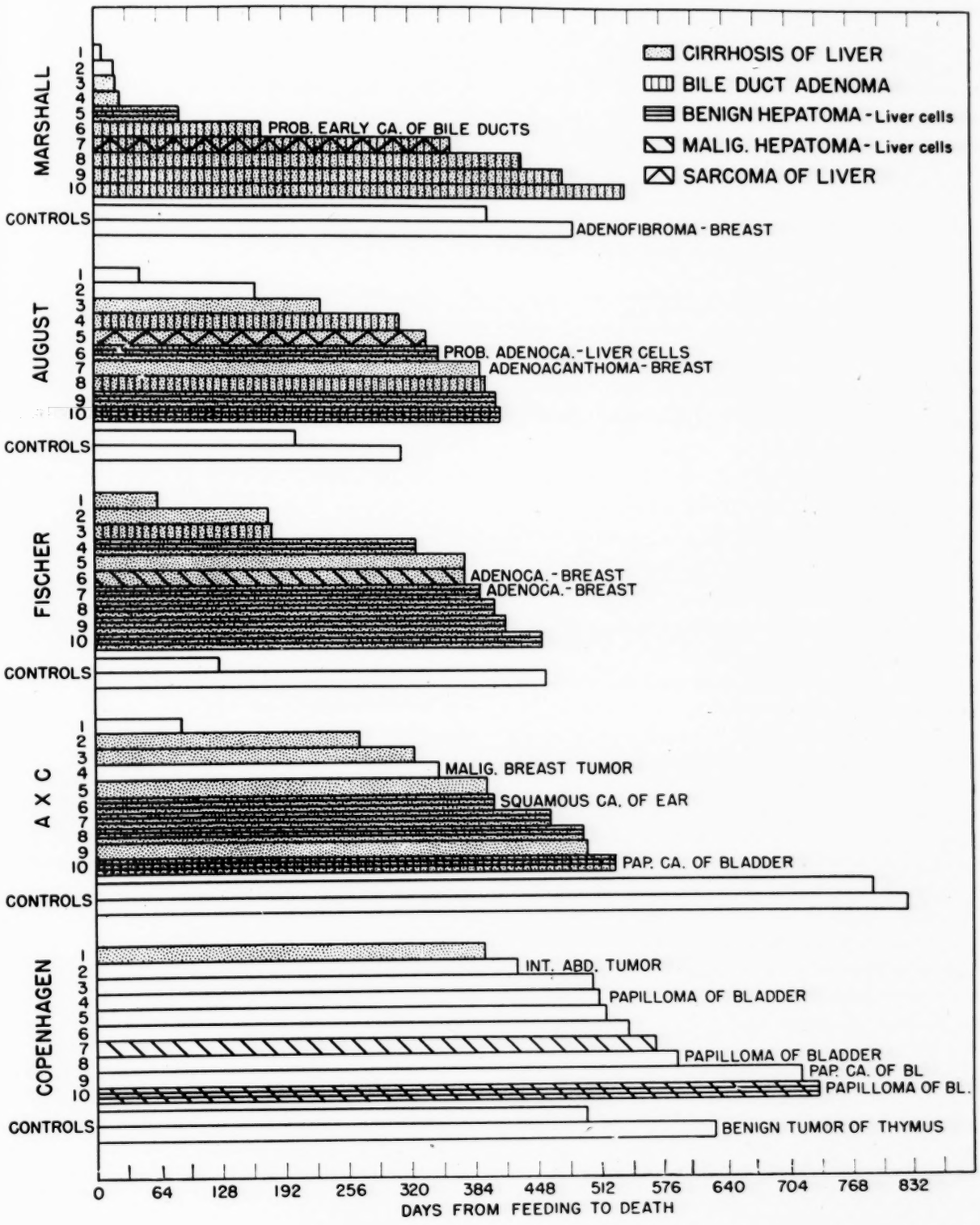


FIG. 1.—Survival period and tumor history for each rat.

RESULTS

The results are summarized briefly in Table II and graphically represented in Fig. 1. The average survival period varied from 7.2 months for the Marshall rats to 18.3 months for the Copenhagens. Lesions of the liver were the most constant finding and they included cirrhosis, hyperplasia of the liver cells and bile ducts and benign and malignant tumors arising from the liver cells, stroma and bile ducts. The morphology of the liver tumors parallels those described by Opie (7) in rats fed butter yellow. Representative types are illustrated in Figs. 2 to 9. The Marshall rats appeared to be the most susceptible to the liver lesions. All of these rats that died after 22 days of acetylaminofluorene feeding had cirrhosis and those that died after 87 days had benign or malignant liver neoplasms or both, all but one of which arose from bile duct epithelium. The Fischer rats also all showed cirrhosis of the liver, the first after 64 days of acetylaminofluorene feeding and all but one, which died after 178 days, had neoplasms. These tumors, with one exception, were of primary liver cell origin. Two August rats died after 50 and 162 days, respectively, with no liver lesions but the remainder had cirrhosis and liver neoplasms including 1 sarcoma, 3 bile duct adenoma and 3 hepatoma. Cirrhosis was not as conspicuous in rats of the A × C strain, but some scarring was found in 8. Four A × C rats had multiple liver cell hepatoma, 2 of which were associated with cirrhosis and one had multiple bile duct adenoma, also (Fig. 8). The Copenhagen rats all out-lived the termination of the acetylaminofluorene feeding. The only rat of this strain in which cirrhosis of the liver was observed died 28 days following the end of the acetylaminofluorene feeding. However, 2 rats, which died 7 months and (Fig. 9) a year respectively after the termination of the experimental feeding, had extensive liver cell cancers. Possibly these rats had previously shown some cirrhosis which was completely healed by the time of their death.

Bladder tumors were observed in 5 rats, 4 of which were Copenhagen and the other was an A × C. These tumors were not associated with calculi as will be described in a later publication for bladder tumors induced by stilbestrol in rats of the Copenhagen and A × C strains. Hyperplasia and thickening of the bladder mucosa was observed in several other rats of these 2 strains and bladder lesions were absent in rats of the 3 other strains.

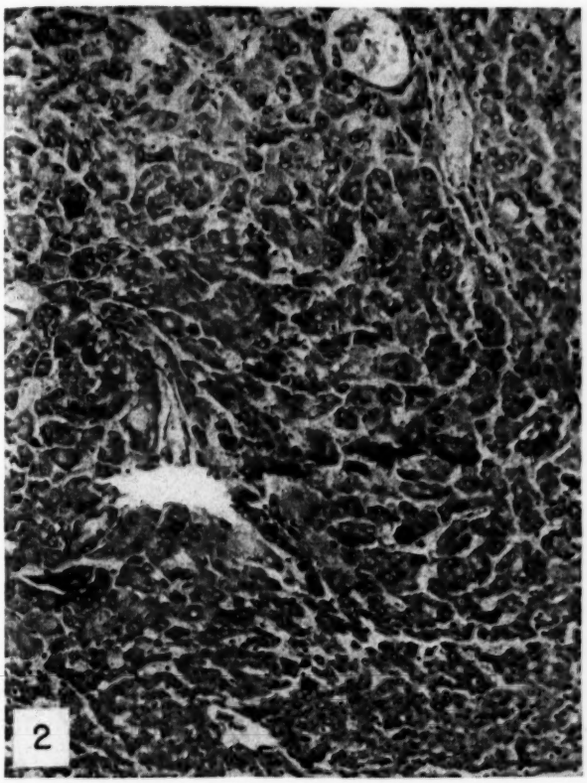
TABLE II: FOR EACH STRAIN THE AVERAGE SURVIVAL PERIOD AND THE NUMBER OF RATS WITH LIVER, BLADDER AND MAMMARY GLAND TUMORS

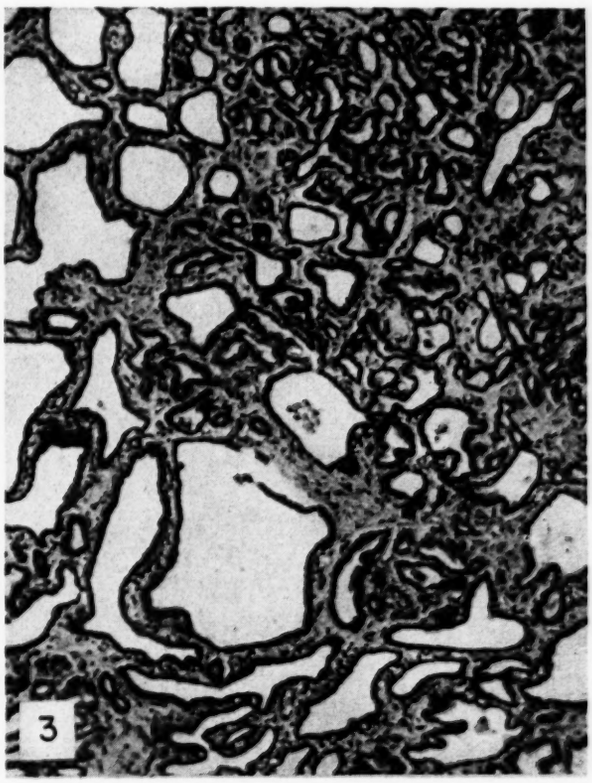
Strain	No. of rats	Age at start, days	Aver. days to death	Liver lesions			Bladder tumors		Mammary gland tumors		Tumors in other locations
				Cirrh.	Benign tumors	Malig. tumors	benign	malig.	benign	malig.	
Marshall	10	120	215	8	6	2	0	0	0	0	0
August	10	110	304	8	5	2	0	0	0	1	0
Fischer	10	100	316	10	7	1	0	0	0	2	0
A × C	10	139	380	8	5	0	0	1	0	1	1
Copenhagen	10	144	549	1	0	2	2	2	0	0	1
Total	50	123	353	35	23	7	2	3	0	4	2
Control	10	127	474	0	0	0	0	0	1	0	1

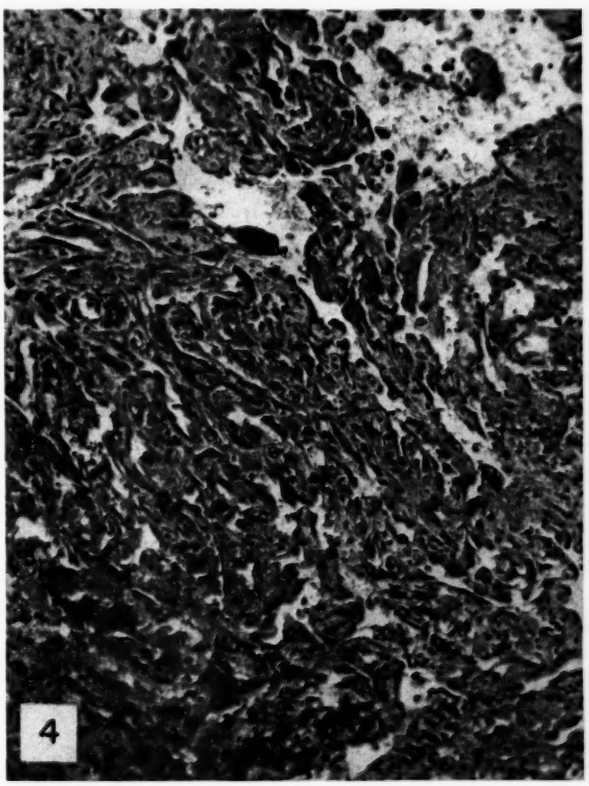
Four malignant mammary gland carcinomas were observed, 2 in Fischer females and 1 each in the August and A × C rats. Fig. 10 shows a section of one of those which were observed in Fischer rats and Fig. 11, the lung metastasis in the one that died 376 days after the beginning of acetylaminofluorene feeding. The morphology of the tumor found in the August rat was exceptional (Fig. 12). This tumor seemed to be composed chiefly of flat epithelial cells arranged in gland-like structures. Malignant mammary tumors rarely occur spontaneously in any of these rats. An earlier analysis (3) showed about 1 in 4,500 untreated females over 11 months of age. Further, these tumors did not appear to be associated with the precancerous lesions frequently observed in rats with stilbestrol-induced mammary cancer. There was no generalized hyperplasia of the mammary tissue or increase in size of the adrenals or pituitary. These factors are being more carefully studied in subsequent series of rats fed acetylaminofluorene in special diets.

One Copenhagen rat had an internal abdominal tumor which was accidentally discarded without determining its location or histogenesis and one A × C rat had a squamous carcinoma of the ear.

The controls, composed of 2 rats of each strain, survived an average of 474 days after the beginning of the experiment or longer than any of the experimental groups except the Copenhagens. No liver or bladder lesions were observed in these rats. One Marshall rat had a benign fibroadenoma of the mammary gland and one Copenhagen had a benign tumor of the thymus, which is fairly characteristic of these rats (4).







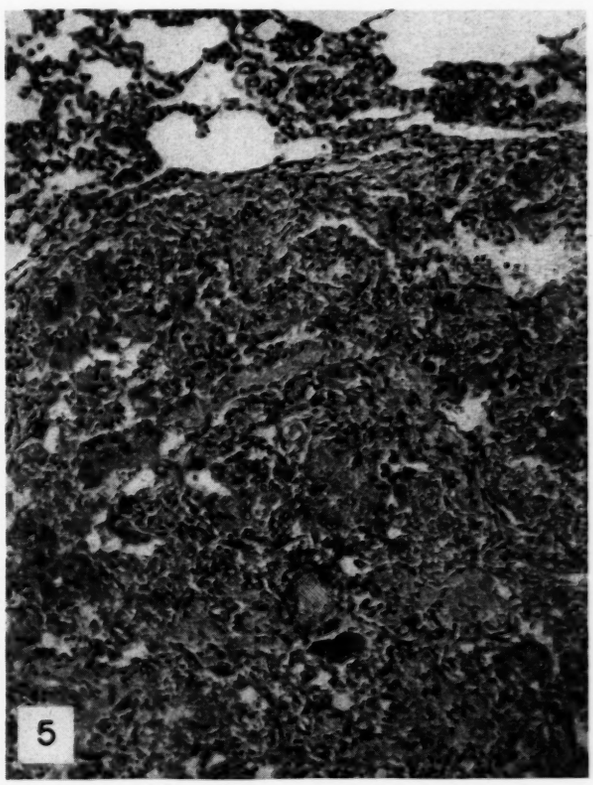
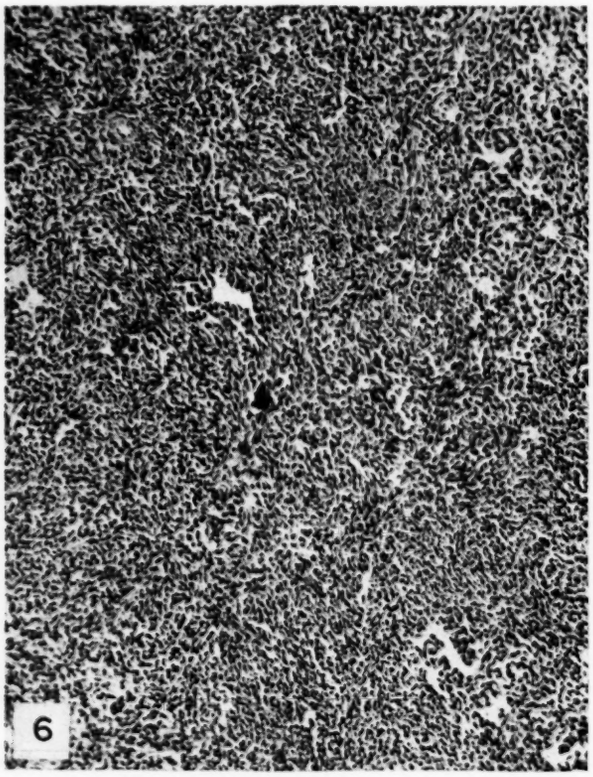


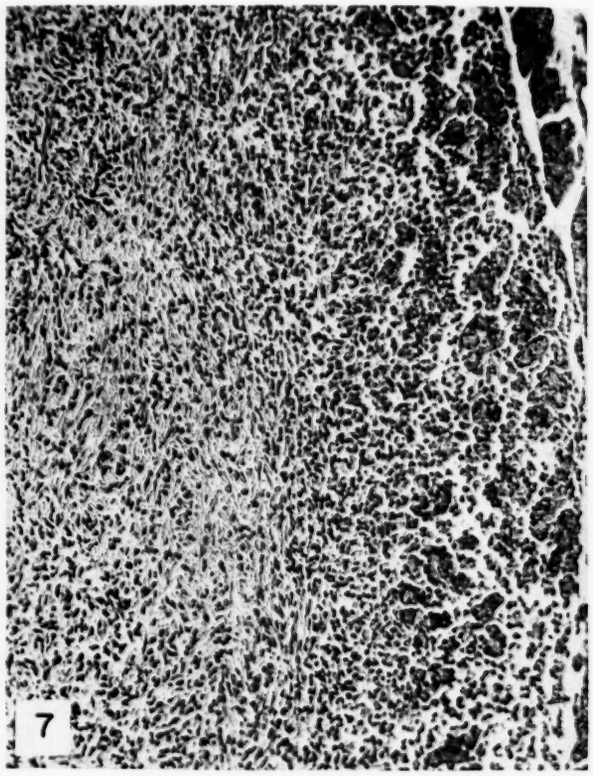
FIG. 2.—Benign hepatoma in A × C female rat 462 days after beginning of acetylaminofluorene feeding. Mag. × 150.

FIG. 3.—Cholangioma in Marshall female rat 174 days after cessation of acetylaminofluorene feeding. Mag. × 150.

FIG. 4.—Malignant hepatoma in Copenhagen female rat 202 days after cessation of acetylaminofluorene feeding. Mag. × 150.

FIG. 5.—Lung metastasis of malignant hepatoma shown in Fig. 4. Mag. × 150.





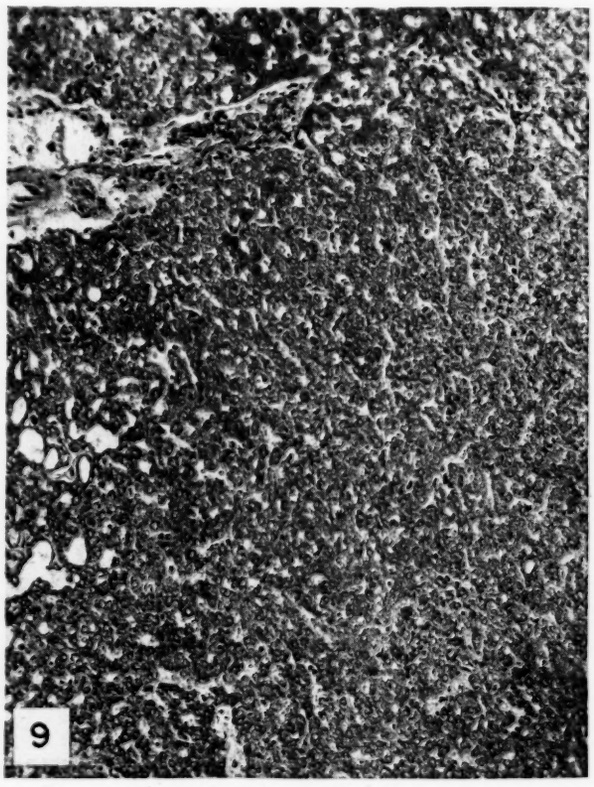
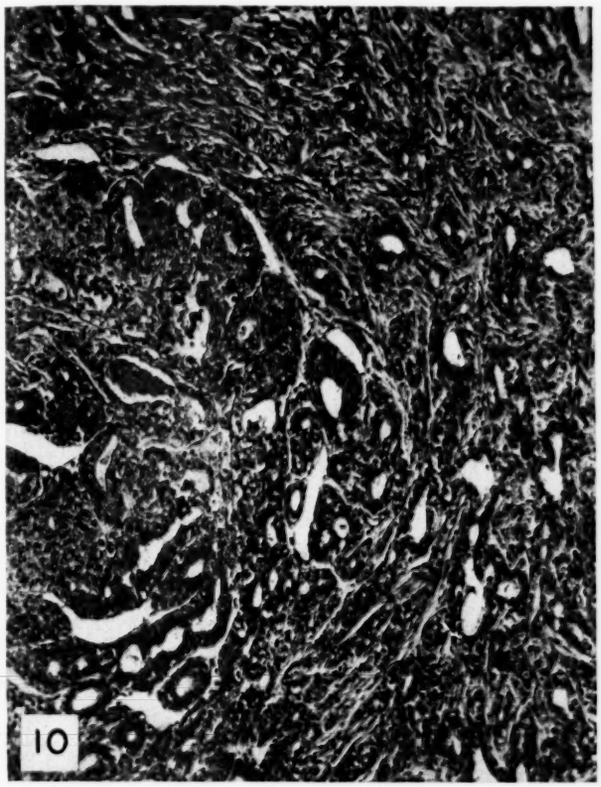


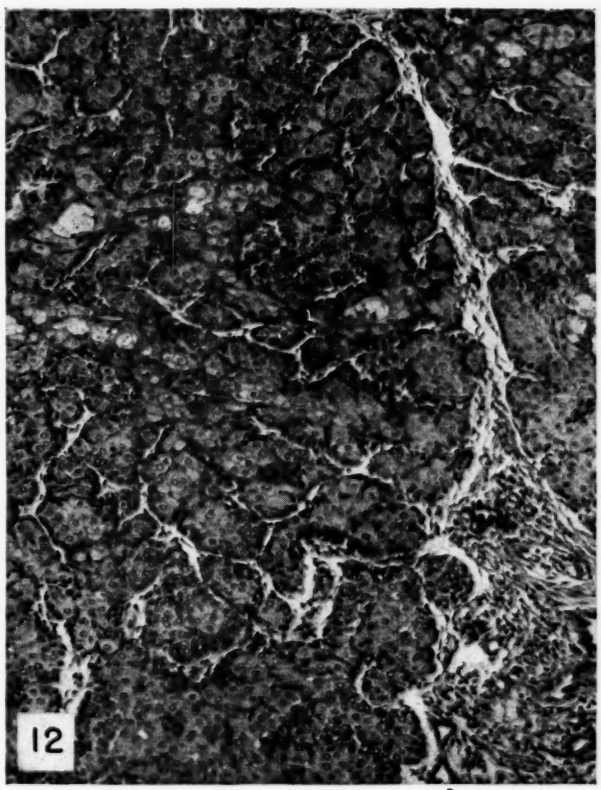
FIG. 6.—Sarcoma in liver of August female rat 366 days after beginning of acetylaminofluorene feeding. Mag. × 150.

FIG. 7.—Metastasis in pancreas of liver sarcoma shown in Fig. 6. Mag. × 150.

FIG. 8.—Benign hepatoma of liver cells and bile duct adenoma in A × C female rat 160 days after cessation of acetylaminofluorene feeding. Mag. × 150.

FIG. 9.—Primary liver cell carcinoma in Copenhagen female rat 369 days after cessation of acetylaminofluorene feeding. Mag. × 150.





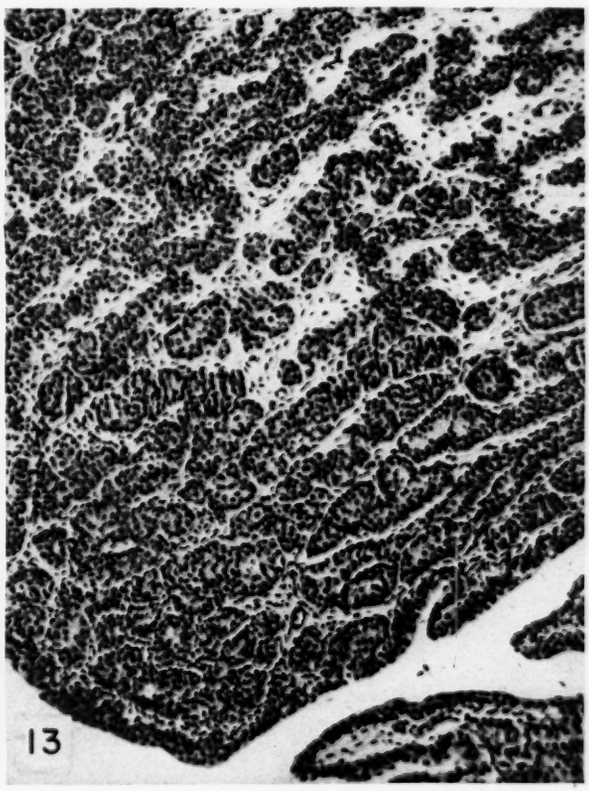


FIG. 10—Adenocarcinoma of mammary gland in Fischer female rat 376 days after beginning of acetylaminofluorene feeding. Mag. × 150.

FIG. 11.—Lung metastasis from mammary adenocarcinoma shown in Fig. 10. Mag. × 150.

FIG. 12.—Adenoacanthoma of mammary gland in August female rat 391 days after beginning of acetylaminofluorene feeding. Mag. × 150.

FIG. 13.—Papillary carcinoma of bladder in A × C female rat 160 days after cessation of acetylaminofluorene feeding. Mag. × 150.

SUMMARY

1. Fifty rats of 5 different inbred lines were fed an average of 3.4 mgm. daily of 2-acetylaminofluorene in Sherman's mash. Forty-one neoplasms were observed, 15 of which were malignant, one not known, and the remainder probably benign.

2. Rats of the Marshall, August, Fischer, A × C, and Copenhagen strains survived an average of 7.2, 10.1, 10.5, 12.7, and 18.3 months, respectively, after the beginning of acetylaminofluorene feeding. The controls lived an average of 15.8 months after the beginning of the experiment.

3. Lesions of the liver were most frequent but not quantitatively associated with the amount of acetylaminofluorene ingested. Cirrhosis was noted in 35 rats including 8 each of 3 strains, all of a fourth and only 1 of the fifth. Twenty-three benign hepatomas and bile duct adenomas were observed, usually associated with cirrhosis. Seven malignant liver neoplasms included 2 sarcoma, 2 carcinoma of bile duct origin and 3 liver cell carcinomas; 2 of the latter showed no cirrhosis in the sections of the liver which were examined.

4. Six of the 8 liver neoplasms observed in Marshall rats were of bile duct origin, while 7 of 8 liver tumors in Fischer rats arose from the primary liver cells. No bile duct tumors were observed in Copenhagen rats, only one in the A × C rats and both bile duct and liver cell tumors were equally represented in the August rats.

5. Bladder papilloma and squamous cell cancer were observed in 5 rats including 4 Copenhagens and 1 A × C. Bladder lesions were absent in rats of the 3 other strains.

6. Malignant mammary tumors were observed in 4 rats including 1 August, 1 A × C and 2 Fischer strain animals.

7. Only 10 rats of each strain were tested but they suggest real constitutional differences in response to this chemical.

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Observations on Radiation-Induced Lymphoid Tumors of Mice*

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(Received for publication October 28, 1946)

INTRODUCTION

The purpose of this paper is to present certain heterogeneous observations recorded during an investigation of leukemogenesis in irradiated mice. Inasmuch as some of this data is a prerequisite to the proper planning of other experiments on induced leukemia, it is felt that this communication may help other investigators in this field to avoid experimental pitfalls.

Radiation has been established as one of the most important exogenous leukemogenic agents in mice (8, 12, 16), and there is evidence suggesting that it exerts a similar influence in man (13, 22, 30). The experimental literature on spontaneous and induced leukemia and lymphoid tumors has been summarized in recent years by Furth (5), Kirschbaum (15), and Engelbreth-Holm (4).

The sequence of events which occurs during the latent period between exposure to the agent and the appearance of the tumor or leukemia is still a subject of speculation. However, a number of factors which control or modify the incidence of the disease have been elucidated, among which may be listed the dosage of the agent (9, 14), the genetic constitution of the mice (16, 23), the nutritional state (18, 28), and the maternal age at parturition (19). Sex differentials in the incidence of lymphoid tumors have been reported in some strains (21, 24) but not in others (17). That the sex hormones play an important role is attested by the fact that estrogens are capable of inducing these tumors in certain strains of mice (9). Moreover, ovariectomy has been shown to decrease, and orchidectomy to increase, their incidence in other strains (21, 24).

However, little is known about the relationship between the age at the time of exposure to the agent and the subsequent incidence of lymphomas. Cowdry and Suntzeff (2) found that the percentage of methylcholanthrene-induced epidermoid tumors varied with age in one strain of mice but not in another. An experiment designed to demonstrate a similar age-dependence in the case of induced lymphoid neoplasms might establish age as a new variable, and might give some clue as to the mechanism of action of the leukemogenic agent. The results of one such experiment are here reported. In view of the strain variation noted by Cowdry and Suntzeff, and in order to further investigate this age-dependence factor, work is now being repeated with larger numbers of mice per age group, a larger series of groups, and a different strain of mice.

The results of two initial experiments intended to determine the influence of thymectomy and splenectomy, respectively, on the incidence of radiation-induced lymphomas are also reported here because the data pertaining to age-dependence render them inconclusive, thus illustrating the importance of controlling and suitably selecting age in designing such experiments. Furth and his co-workers (6, 21) found that thymectomy greatly reduced the incidence of spontaneous lymphoid tumors in strain Ak mice, while splenectomy had no influence. The data to be reported here are equivocal but would suggest that removal of the thymus slightly decreased the incidence and markedly delayed the appearance of radiation-induced lymphomas, while splenectomy reduced the incidence but did not affect the latent period.[1]

Incidental observations pertaining to the sites of origin of these neoplasms, their probable unicentric initial development, and their dissemination through the body are also presented.

MATERIALS AND METHODS

The age-dependence study will be referred to as Experiment I, that on thymectomy as Experiment II,

* These investigations have been aided by a grant from The Jane Coffin Childs Memorial Fund for Medical Research.

[1] The term "latent period" is used synonymously with "induction time" here, and both terms refer to the lapse of time between the first x-ray exposure and the observation of a grossly detectable lymphoid tumor, either at autopsy or at the onset of pronounced dyspnea in a subsequently autopsied mouse.

and that on splenectomy as Experiment III. Strain A mice, initially obtained from Dr. L. C. Strong and subsequently bred in the laboratory, were used for all experiments. In previous work with this strain, the incidence of spontaneous lymphoid tumors in 80 untreated animals was 3.8 per cent; no lymphoid tumors were observed in 55 animals treated with methylcholanthrene (16). Spontaneous lymphomas are quite rare in strain A mice less than 1½ to 2 years old.

In Experiment I, a total of 198 mice, equally divided by sexes, were irradiated by groups at 2 weeks, 1 month, and 2, 3, 4, and 6 months of age, respectively. Roentgen radiation was given in fractionated, daily doses of 50 r on 12 consecutive days for a total of 600 r of whole body radiation; the physical factors were 125 K.V.P., 6 Ma., 3 mm. Al filter, 30 cm. distance, 43 r per minute. The mice were placed in perforated cardboard boxes measuring 5 × 6 × 1½ inches which were then carefully positioned for treatment, each box containing 5 to 7 mice. Deaths attributable to irradiation occurred in a small number of animals in each group except for the 2 week old mice, which succumbed in such large numbers that an inadequate residue remained for observation. Following irradiation, the mice were maintained with no further experimental treatment and observed for the development of lymphoid tumors. All mice surviving approximately 1 year after irradiation were killed and the experiments terminated. The maximal age of the animals was therefore 12 to 18 months. During this period the mice were caged in groups of 4 to 6 and received Purina fox chow checkers and water *ad libitum*.

Experiments II and III were set up concurrently with Experiment I, and the same roentgen dosage was employed. The mice in these experiments, however, were all 2 to 3 months of age at the time of irradiation. A total of 73 mice were thymectomized, under ether anesthesia, by the method of Segaloff (29). Of these, 35 were operated upon 1 week before irradiation, and 38 were subjected to thymectomy about 1 month after the end of x-ray exposure. Similarly, in Experiment III, 36 mice were splenectomized one week before irradiation, and 48 others 1 month after, for a total of 84 animals. The controls for these experiments served also as the 2 and 3 month age groups of Experiment 1. Both sexes were equally represented among the animals surviving through the latent period. The diet and general environment paralleled the conditions of Experiment I.

RESULTS

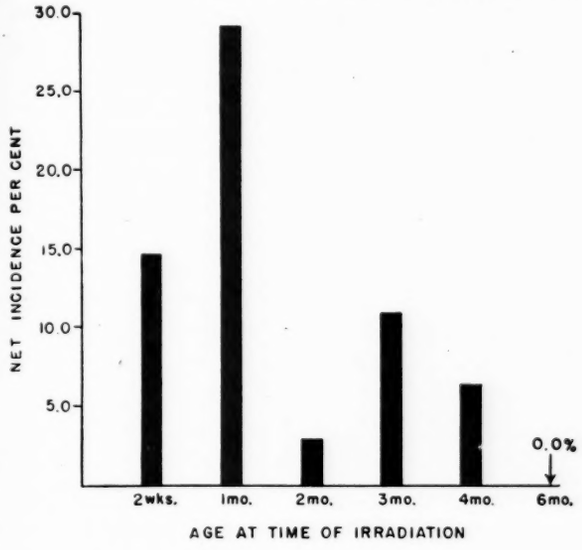
The results of Experiment I are summarized in Table I, together with incidental observations on the occurrence of ovarian and mammary tumors, and the net incidence of lymphoid tumors by age groups is graphically presented in Fig. I. It can be seen that the greatest incidence of lymphoid tumors occurred in the group irradiated at one month of age, and that there is a sharp drop between 1 and 2 months. The number of tumors developing in the 1 month group was greater than the combined total among all of the older age groups. There were 10 lymphomas among 38 surviving animals treated at 1 month of age or younger, an incidence of 26.4 per cent, and only 7 tumors (6.0 per cent) among 117 mice 2 months of age or over at the time of irradiation. The average induction time for the entire series of 17 tumors was 215.0 days. The 10 tumors in the combined younger group had an average induction time of 207.9 days, against an average of 225.4 days in the older age groups. The minimum induction time was 157 days, in a mouse of the 1 month old group, and the maximum, 301 days, in a mouse of the 3 month old group. Ten of the 17 tumor-bearing mice were males, and 7 were females. This sex difference is not considered significant.

TABLE I: AGE AND SEX IN RELATION TO LYMPHOMA INCIDENCE

	Age at onset of irradiation						Total		
	2 wks.	1 mo.	2 mos.	3 mos.	4 mos.	6 mos.	♂	♀	Both
Total initial No. of mice	23	23	38	54	20	30	99	99	198
Deaths within 4 months after irradiation	16	2	3	8	4	10	21	22	43
Net No. of mice	7	31	35	46	16	20	78	77	155
Lymphoid tumors									
No.	1	9	1	5	1	0	10	7	17
Net incidence, %	14.3	29.0	2.8	10.9	6.2	0.0	12.8	9.1	11.0
Ovarian tumors									
No.	0	6	1	2	1	0		10	
Mammary tumors									
No.	0	0	0	1	2	1		4	

Of 73 thymectomized and irradiated mice, a total of 45 (22 males, 23 females) survived for at least 5 months after radiation. Only 2 surviving mice of this group (4.2 per cent) developed lymphoid tumors, with induction times of 379 and 437 days, respectively. One of these had been thymectomized prior to x-ray treatment; the other, 1 month after radiation. No residual or

FIG. 1. NET INCIDENCE OF LYMPHOID TUMORS BY AGE GROUPS



recurrent thymic tissue was found at autopsy in control or experimental animals. The results in the thymectomized animals must be compared with those in intact irradiated controls of the same age at the time of treatment.[2] The combined incidence for the 2- and 3-month old control groups was 7.4 per cent, and the latent periods ranged from 162 to 301 days, with an average of 229 days. The difference in incidence is of doubtful significance, but the difference in induction time appears quite pronounced. Ten thymectomized and irradiated mice are still alive more than a year after irradiation, and it is possible that additional tumors may still appear in this small group. Thymectomized but nonirradiated controls were omitted because of the very low incidence of spontaneous lymphomas in nonirradiated mice of this strain. Likewise, in Experiment III, no splenectomized, nonirradiated mice were maintained.

In Experiment III, 57 mice (29 males, 28 females) of an initial total of 84 survived at least 5 months, and only one of these (1.7 per cent) developed a lymphoma 231 days after treatment. Using the control data cited in the paragraph above for comparison, there appears to have been a slight decrease in incidence, of questionable significance, without a corresponding increase in induction time following splenectomy. It is apparent, however, that results of greater significance might have been obtained had the age been so selected as to yield a maximal number of tumors in the analogous controls. Unfortunately, the age-dependence data were not available when these experiments were initiated, and the marked discrepancy between the incidence of the 1- and 2-month-old groups was not suspected. These experiments are now being repeated using younger mice of strains A and C57 black.

The tumors were all lymphomas of the stem-cell, lymphoblastic, or lymphocytic varieties, apparently identical with those induced by methylcholanthrene (20) or by estrogens (9). No myeloid or monocytic forms were seen. The thymus is by far the organ most commonly involved. The spleen is distinctly less often infiltrated, and certain non-lymphoid organs (the lung, liver, kidney, heart, and adrenal, in order of frequency) are invaded more often that the superficial lymph nodes. These nodes, which are usually involved early in the course of the spontaneous disease, are often completely devoid of lymphoma, both macroscopically and microscopically, in the radiation-treated animals. The mediastinal lymph nodes are not uncommonly invaded by direct extension from the thymus, and the mesenteric nodes are distinctly more often affected than the superficial nodes. There is invasion of the peripheral blood to give a true leukemia in a considerable number of instances, and white blood cell counts as high as 800,000 have been observed. The exact percentage of tumor-bearing mice in which leukemia becomes manifest is unknown because the disease was undetected until autopsy in a number of instances. The brain and testis have rarely been infiltrated in the animals observed to date; in 2 animals, focal collections of tumor cells were noted in the meninges. Several of the tumors have been successfully transplanted, by subcutaneous, intravenous, intraperitoneal, or intraocular inoculation, into other strain A mice, F_1 hybrids of strain A parentage, and rarely into Bagg albino mice. They have not grown on inoculation into the anterior chamber of the guinea pig eye, following the technic of Greene (11).

The route of dissemination of the disease is not clear. It might be surmised, from the fact that frank leukemia occurs not uncommonly, that the tumor enters the blood stream and reaches distant organs by the hematogenous route. However, histological study reveals that the tumor collections in the lungs occur in and ultimately occlude the peribronchial and perivascular lymphatic channels (Fig. 4), and secondarily spread to the subpleural lymphatics; parenchymal pulmonary

[2] These controls also served as the 2- and 3-month-old groups of Experiment I, and as intact controls for Experiment III.

infiltration, either in the form of massive consolidations packed with tumor cells or as focal aggregations in the alveolar walls, is much less common. Similarly, the perivascular lymphatic spaces of the hepatic portal triads are the most common site of involvement in the liver, occasionally accompanied by tumor-cell collections invading the sinusoids of the parenchyma. When the architecture of the spleen has not been completely obliterated by tumor, the white pulp is seen to be invaded first. In the kidney, the glomeruli are rarely involved; the peritubular zones of the cortex and the subepithelial lymphatic spaces of the pelvis are the usual sites of infiltration, at times accompanied by massive subcapsular and perirenal accumulations which may secondarily invade the periphery of the adrenal or ovary. It would appear, therefore, that the lymphatic route also plays an important role in the dissemination of these tumors.

Controversy has long existed as to whether lymphatic leukemia and lymphoid tumors represent a primarily systemic disease, originating simultaneously in multicentric foci, or an initially unicentric neoplastic proliferation which later disseminates. Potter, Victor, and Ward (26), working with spontaneous leukemia in strain C58 mice, collected evidence suggesting that the disease may be unicentric, usually preceded by reticular hyperplasia and lymphopoiesis which varied in degree in successively biopsied lymph nodes. McEndy, Boon, and Furth (20) studied the early stages of leukemogenesis in mice of the Rf stock which had been painted with methylcholanthrene. They found that a rather abrupt malignant change takes place in the lymphoid tissues with the apparently simultaneous appearance of large, atypical mononuclear "tumor" cells in the spleen and lymph nodes. When these cells were too sparsely present to support a histological diagnosis of leukemia in lymph node or spleen sections, bioassay of other tissue fragments from the same node or spleen frequently confirmed the malignant change. They concluded that leukemia induced by methylcholanthrene results from an abrupt malignant change in multiple, widely scattered cells of the lymphoid series.

[3] The terms "monocentric" and "unicentric" are used loosely to denote origin in a single lymphoid organ and not in the strict sense of origin in a single focus within an organ.

The predominant and early involvement of the thymus in certain spontaneous instances and in most of the estrogen- or x-ray-induced tumors would in itself suggest that the thymus may be the site of a monocentric origin of the disease.[3] This thesis received additional support when McEndy, Boon, and Furth (21) demonstrated that thymectomy greatly reduced the incidence of spontaneous leukemia in mice of the Ak stock, but that splenectomy had no such effect. In the course of the present investigation, several animals have been killed near the end of the latent period because of dyspnea, and a gross examination revealed no abnormalities except for minimal enlargement of the thymus and perhaps a slight change in its consistency. On histological examination, however, one lobe of the thymus was seen to be completely replaced by a lymphoma while the adjacent lobe exhibited intact architecture and no definite evidence of infiltration (Figs. 2 and 3). All of the other organs, including the lymph nodes and spleen, were apparently free of disease. These chance observations clearly indicate that a lymphoid tumor may arise and proliferate in one lobe of the thymus at a time when the other lobe, and all of the other tissues, are histologically uninvolved. In another instance, one lobe of the thymus was of normal size, the opposite lobe was enlarged and lymphomatous, and two adjacent lymph nodes were intensely hyperplastic but apparently contained no tumor. That the thymus is merely the most susceptible and not the sole site of origin is also indicated by the infrequent development of primarily splenic lymphomas in both intact and thymectomized mice. To date, no instance of a radiation-induced lymphoma apparently originating in a peripheral lymph node has been observed. Bioassay studies during the latent period, using the thymus glands of irradiated mice, should yield additional information concerning the problem of the unicentric origin of lymphomas.

INCIDENTAL OBSERVATIONS

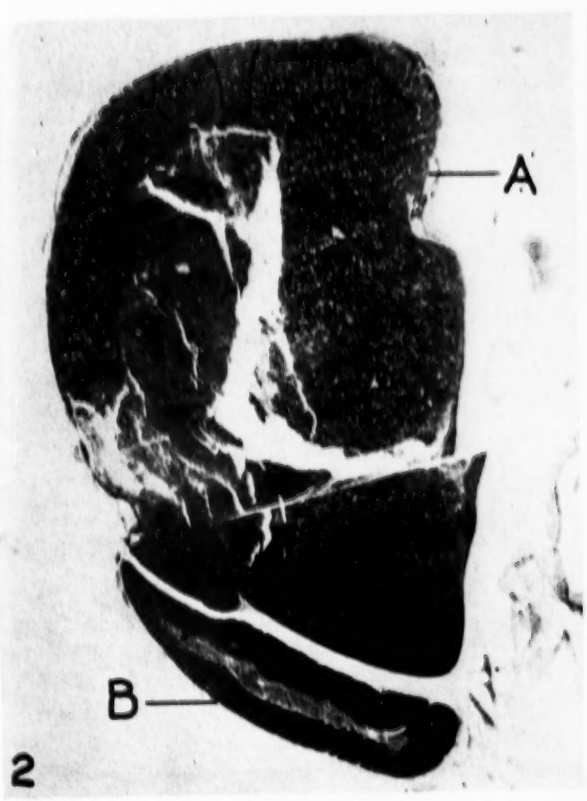
The development of ovarian tumors after irradiation of mice has previously been reported by Furth and his co-workers (7, 8). Ten mice in Experiment I developed tumors of the ovary which were bilateral in four instances. Additional ovarian neoplasms might

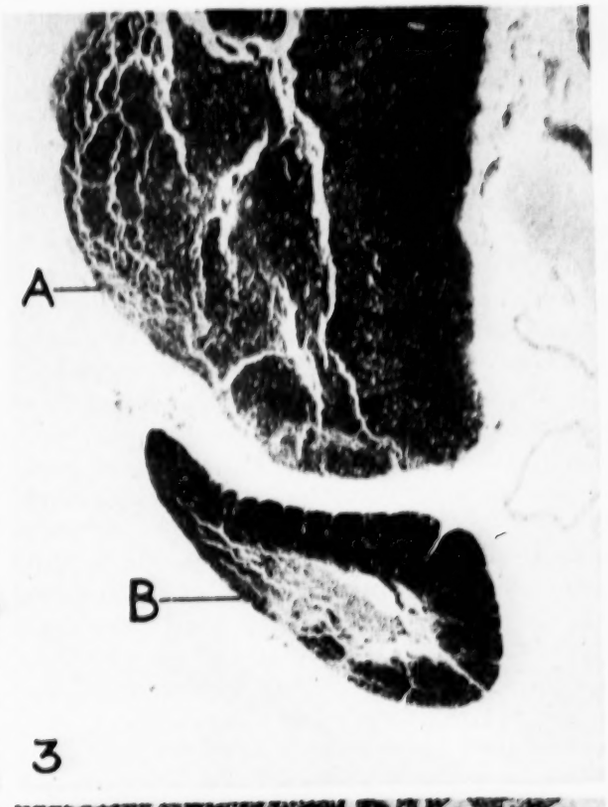
DESCRIPTION OF FIGURES 2 TO 5

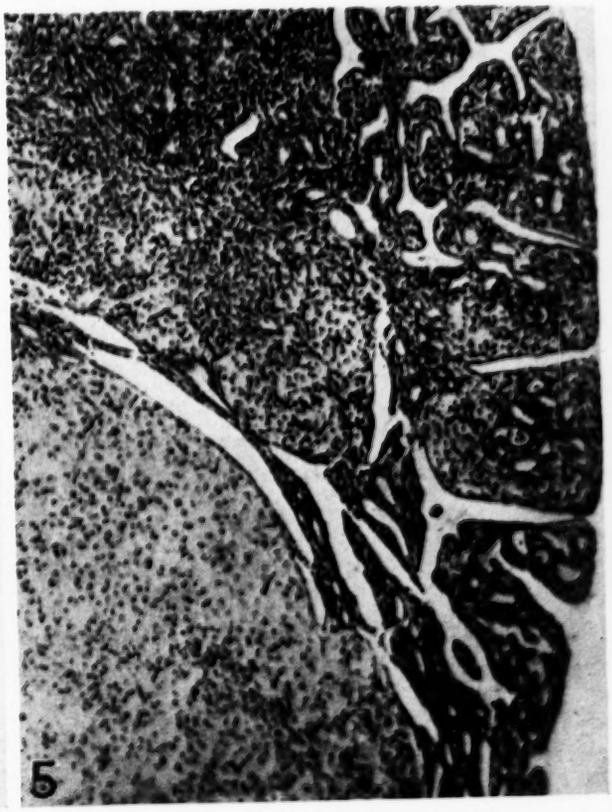
FIGS. 2 and 3.—Sections through the thymus reveal, in each case, a normal lobe (B) and a greatly enlarged lobe (A) which is replaced by a lymphoid tumor. In both instances the other lymphoid and visceral tissues were not involved.

FIG. 4.—Low-power magnification of the lung revealing dark areas of lymphoma in the peribronchial spaces, and a subpleural tumor nodule.

FIG. 5.—Ovarian tumor of the luteoma variety showing cells with small, round, pale nuclei and extremely pale cytoplasm, above which is an adenomatous type of tubular proliferation mixed with some lutein cells.







FIGS. 2 TO 5

have been expected if the experiment had been maintained longer. Six of these 10 mice had been irradiated at 1 month of age, suggesting that age may also modify the incidence of radiation-induced ovarian tumors. These neoplasms, occurring in anovular ovaries, were predominantly composed of sheets of cells having a small round nucleus and rather large amounts of clear cytoplasm; this type has been previously designated as a luteoma (Fig. 5). Less often, atypical granulosa cell tumors have been observed. In a number of instances, these two cellular types have occurred side by side in the same ovary. No metastases have been observed to date. The latent period has been approximately 6 to 9 months for most of these neoplasms.

Breeding female mice of Strain A, having the milk influence, are highly susceptible to the development of spontaneous mammary tumors. The incidence of these tumors is sharply reduced by irradiation. In Experiment I, only females that were old enough to be breeding at the time of irradiation (the 3 to 6 month age groups) bore any of these tumors, and the total incidence was only 4 in 77 surviving females, or approximately 3 per cent. Only 1 animal had both an ovarian and a mammary tumor. Whether the inhibition caused by roentgen radiation was due not only to sterilization but in part also to diminution of circulating levels of estrogen is not known. It is well known that after doses of x-rays adequate to destroy all ovarian follicles, estrogen production persists in quantities adequate to permit the continuation of estrus cycles (10, 25). It is probable, however, that some decrease in estrogen secretion occurs after radiation.

DISCUSSION

The results presented are preliminary and do not warrant extensive discussion at this time. The age dependence of radiation-induced lymphoid tumors, if verified on reinvestigation, would suggest some interesting implications. The apparent critical period of susceptibility is somewhere between 1 and 2 months of age, which, in a mouse, roughly corresponds with the time of puberty. Inasmuch as puberty is a period of intense endocrine activity, it is possible that radiation at this time can more easily disturb the inter-relationships of the endocrine glands and throw their activities out of balance. It is known that endocrine imbalance contributes to the development of other neoplasms, and that the lymphoid tissues are under hormonal influences, particularly those of the adrenal and pituitary (3), gonads, and thyroid (27). Andreasen (1) studied the development and involution of the lymphoid organs of the white rat in relation to age, and found that the thymus reached its maximum size at 2 to 3 months of age, or approximately at the time of puberty. The alternate possibility cannot yet be excluded that the high incidence at or about the time of puberty is due merely to the presence of a greater mass of lymphoid tissue in the thymus at this age.

SUMMARY

1. Strain A mice in groups of various ages yielded a maximum incidence of lymphoid tumors in animals irradiated at 1 month of age with a sharp decrease at 2 months and later.

2. Thymectomy of strain A mice irradiated at 2 to 3 months of age resulted in a considerable increase in the latent period and a very slight decrease in the incidence of lymphomas as compared with the low incidence in intact irradiated control mice of the same age. In a parallel experiment, splenectomy was followed by a slight decrease in incidence without change in latent period.

3. The tumors are lymphocytic lymphomas which, in most instances, appear to arise in the thymus, to disseminate to the spleen, lungs, liver, kidneys, and lymph nodes, and to cause leukemia in a considerable percentage of animals.

4. Incidental observations on radiation-induced ovarian tumors and on inhibition of spontaneous mammary tumors following x-ray treatment are presented.

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Changes in the Succinoxidase Activity of Livers from Rats during the Development of Hepatic Tumors on Feeding *p*-Dimethylaminoazobenzene

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(Received for publication September 9, 1946)

In a series of papers, Salter and his collaborators (3, 11, 16) reported the results of their investigation of the respiratory activity of tumors of various origins in the presence of succinate or *p*-phenylenediamine as substrate. They took the oxygen uptake of homogenate or slices in a Ringer-glucose medium as basic oxygen consumption and found in tumor tissues a very small response to the addition of these substrates. The response in homologous normal tissue was much higher. These authors consider that the lowered capacity to oxidize the above substrates in tumor tissue "might be a useful adjunct to routine morphological investigation" (3). Rosenthal and Drabkin (15) point out that different normal tissues differ widely in their capacity to oxidize succinate or *p*-phenylenediamine and one might expect a difference between neoplastic and normal parent tissue only in cases when tumors arise in a tissue with normally high succinoxidase content. Detailed studies of the components of the succinoxidase system of tumors and normal tissues were carried out by Potter and his associates. DuBois and Potter (4) found that the cytochrome *c* is diminished in the tumor but unchanged in precancerous tissues. Using homogenized tissues Schneider and Potter (18) found a fairly constant succinic dehydrogenase and cytochrome oxidase content in 10 kinds of experimental tumors. These values were lower than those of normal tissue of high succinic dehydrogenase and cytochrome oxidase content but some normal tissues such as lung or spleen had activities even lower than those of tumor tissues. Liver tumors had only about one-fourth of the succinic dehydrogenase activity and about one-third of the cytochrome oxidase activity of normal liver.

Since Warburg's (19) discovery of the high aerobic glycolysis of tumor slices, theories have repeatedly been brought forward to explain carcinogenesis in terms of changes of enzymatic processes. A new impetus to these theories came from the work of Kensler and his group (10) who demonstrated that substances which were isolated from urine of rats fed *p*-dimethylaminoazobenzene (17) depress the activity of the diphosphopyridine enzyme system. Potter (13) found an inhibition of the succinic dehydrogenase system by similar substances. Potter (14) advanced a theory that cancer may be the result of a competition between a hypothetical enzyme (X) and a derivative thereof arising from it by the action of carcinogenic agents. While confirming the inhibition of the succinoxidase activity in normal liver tissue by aromatic diamines of the same type as the metabolites of *p*-dimethylaminoazobenzene, Elson and Hoch-Ligeti (6) established the fact that no depression by these aromatic diamines occurred in the succinoxidase activity of tumor tissue, or normal tissue of low metabolic activity. On the contrary, a prolonged increase of the O_2 uptake was observed in such tissues, due to slow oxidation of the diamines. It seemed possible that, as a consequence of treatment with *p*-dimethylaminoazobenzene, circulating metabolites might at first depress the high succinoxidase activity of normal liver. On further treatment with *p*-dimethylaminoazobenzene they might not be dealt with further as in normal tissue and so by cumulative action might introduce changes leading to neoplasm. It seemed, therefore, of interest to follow up the changes of the succinoxidase activity of livers from rats from the beginning of the treatment with *p*-dimethylaminoazobenzene until the development of tumors, and of the tumor tissue itself. Since the effect of the diet on the production of liver tumors with *p*-dimethylaminoazobenzene is well established the effects of the different diets on the succinoxidase of the liver were also studied.

EXPERIMENTAL

Albino rats of both sexes were used weighing about 100 gm. at the beginning of the experiment. They

were housed in the same room, in cages containing not more than 10 rats of the same sex. The diets were as shown in Table I. *p*-Dimethylaminoazobenzene in amount of 0.06 gm. was added in 2 ml. arachis oil to 100 gm. of the diet. The rats were allowed to consume the food and water *ad libitum*. The food consumption was higher in males and varied with the different diets, even in the absence of *p*-dimethylaminoazobenzene. It was highest, 12 to 16 gm. per day, in the groups of males receiving milk with diets not containing *p*-dimethylaminoazobenzene. On addition of *p*-dimethylaminoazobenzene to this diet the consumption dropped to about 6 to 8 gm. per day and was similar to that of animals on the basic rice diet. On addition of *p*-dimethylaminoazobenzene no further reduction of the food intake occurred on these diets without milk. Details of food intake and body weight are given elsewhere by Hock-Ligeti (7). The animals on the high-fat diet consumed about 10 gm. per day without, and 7 gm. per day with, addition of *p*-dimethylaminoazobenzene. Animals were sacrificed from the third day onwards at different intervals; the oldest rat was one killed on the 460th day of the experiment. Rats suffering from any disease other than tumors were discarded. The livers were removed as quickly as possible, weighed, and the succinoxidase content determined simultaneously in slices and in homogenates. Warburg-type manometers were used. The experiments were carried out at 38° C. Readings were taken every 5 minutes. Homogenate was prepared in M/30 phosphate buffer according to Potter and Elvehjem (12). The reaction mixture consisted of 1 ml. homogenate containing 40 mgm. of tissue; 0.5 ml. of 6 per cent succinate in M/10 phosphate buffer (pH.-7.35), 0.2 ml. of 1 per cent cytochrome *c* prepared according to Keilin and Hartree (9), 1 ml. M/10 phosphate buffer pH.7.35 and H_2O to make 3 ml. The thickness of the slices was 0.2 to 0.3 mm., the area about 50 sq. mm. and the final dry weight varied from 2 to 8 mgm. In experiments with slices 0.2 ml. of 20 per cent KOH solution was placed in the central cup and the volume of the homogenate was replaced by an equal volume of M/30 buffer. The gas phase was air, or in experiments with slices, oxygen. All experiments with homogenate were carried out with and without addition of an excess of cytochrome *c*. The addition of an excess of cytochrome *c* always resulted in an increase of the succinoxidase activity of the homogenate. No effect of this addition to the slices was found; this result is no doubt due to the fact that cytochrome c does not penetrate through intact cell membranes.

Forty milligrams of wet tissue per vessel gave oxygen uptakes which were not influenced by additions of aluminium and calcium, the use of which has been suggested by Horecker and his co-workers (8) and

TABLE I: ARRANGEMENT OF THE EXPERIMENT AND COMPOSITION OF THE DIET

Diet	Sex of animals	No. of animals	Body weight during the experiment	Caloric value of the diet: Total calories in 100 gm. of food	Caloric value of the diet: Percentage caloric value of the diet in: Carbohydrate	Protein	Fat
Rice, carrots + 2% arachis oil	♂	10	maintained				
" " " " "	♀	10					
Rice, carrots + 2% arachis oil + 0.06% *p*-DAB[1]	♂	10	slightly decreased	412	86.9	6.8	6.3
" " " " " " "	♀	20	" "				
Rice, 6% casein + 2% arachis oil + carrots	♀	10	maintained				
Rice, 6% casein + 2% arachis oil + carrots + 0.06% *p*-DAB	♀	30	"	436	82	12	6
Rice, 10 ml. milk + 2% arachis oil	♂	30	increased				
" " " " " "	♀	20					
Rice 10 ml. milk + 2% arachis oil + 0.06 % *p*-DAB	♂	30	"	477	78.6	8.7	12.7
" " " " " " " " "	♀	20					
" " " " " " " " "	♂	10	"				
" " " " " " " " "	♀	20					
Rice, 10 ml. milk only	♂	20	"	460	81.6	8.9	9.5
" " " "	♀	10					
Rice, carrots	♀	10	maintained	396	90.5	7.1	2.7
Rice, carrots + 2% arachis oil + 0.06% azobenzene	♀	10	"	412	86.9	6.8	6.3
17% Protein, 30% fat	♀	10	increased				
17% Protein, 30% fat + 0.06% *p*-DAB	♀	20	"	480	53	17	30
" " " " " "	♂	10					

[1] *p*-DAB = *p*-dimethylaminoazobenzene.

Axelrod and his group (1) (Table II). By using less tissue the oxygen uptake per unit weight of homogenate was in many cases less than that found when 40 mgm. was used. On addition of aluminium and calcium the relation of oxygen uptake to the amount of tissue became generally linear, but in some cases when 10 mgm. of tissue was used with the addition of these ions the oxygen uptake per unit weight was much higher

TABLE II: EFFECT OF THE ADDITION OF 0.2 ML. 4×10^{-3}M $AlCl_3$ AND 0.2 ML. 4×10^{-3}M $CaCl_2$ ON THE SUCCINOXIDASE ACTIVITY OF 40 MGM. RAT LIVER HOMOGENATE

µl Oxygen uptake per gram wet tissue per hour

No. of Experiment	No cytochrome *c* added		Cytochrome *c* added	
	Without $AlCl_3$-$CaCl_2$	With $AlCl_3$-$CaCl_2$	Without $AlCl_3$-$CaCl_2$	With $AlCl_3$-$CaCl_2$
1	5,500	5,100	7,450	7,100
2	10,000	10,300	11,000	11,000
3	8,150	8,150	10,100	10,100
4	6,400	6,350	7,800	8,000
5	7,400	7,400	8,400	7,800
6	4,890	4,900	6,200	6,200
7	7,000	7,300	....	....
8	10,000	10,500	11,800	10,000
9	6,800	7,200	7,800	8,000

TABLE III: EFFECT OF THE ADDITION OF 0.2 ML. 4×10^{-3}M $AlCl_3$ AND 0.2 ML. 4×10^{-3} M $CaCl_2$ ON THE SUCCINOXIDASE ACTIVITY OF VARYING AMOUNTS OF RAT LIVER HOMOGENATE

µl. Oxygen per gram wet tissue per hour (Excess of cytochrome *c* added)

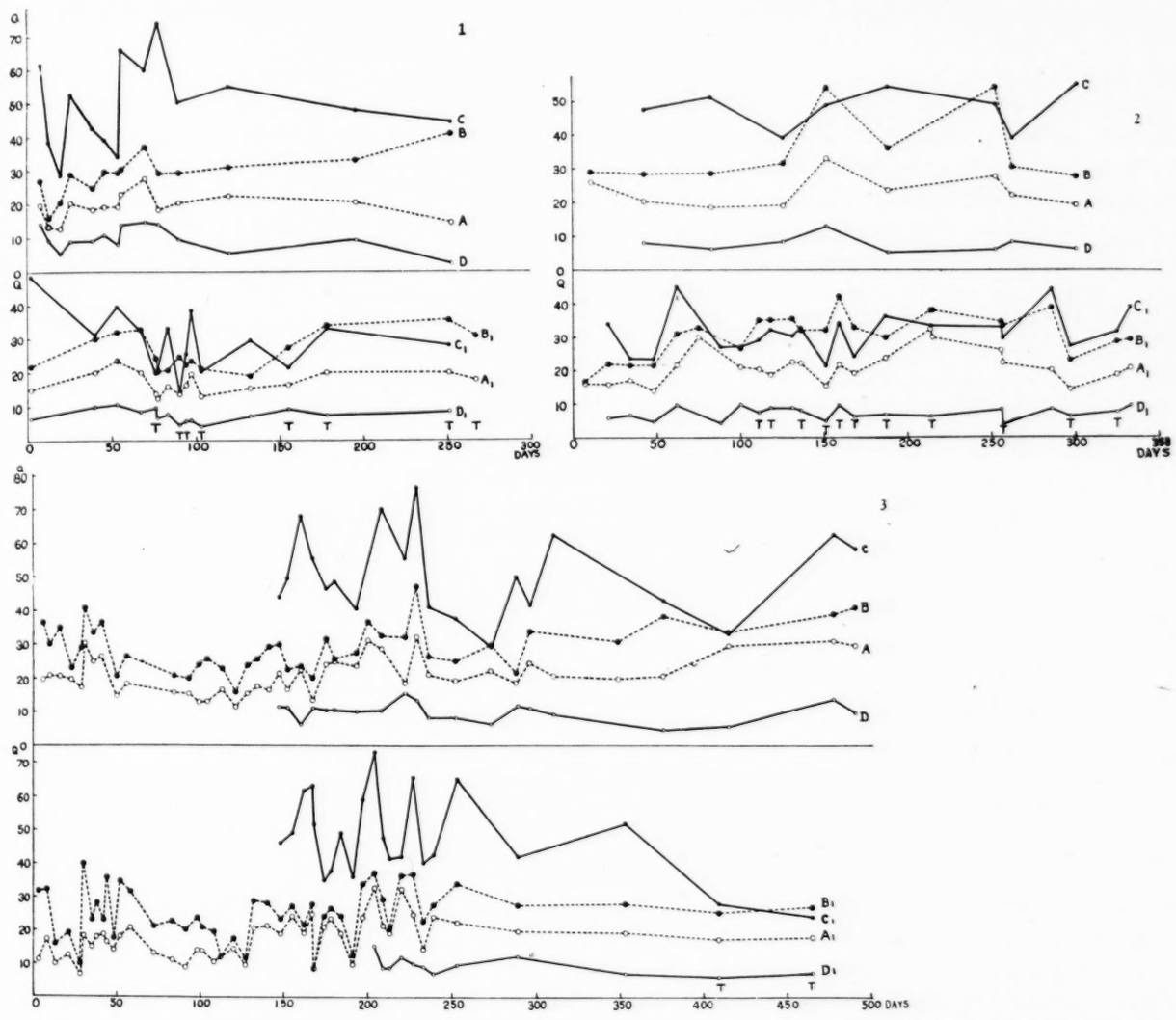
No. of Exper.	Amount of tissue employed, mgm.	Without $AlCl_3$-$CaCl_2$	With $AlCl_3$-$CaCl_2$
1	40	10,000	9,800
	20	3,700	9,600
	10	0	10,300
2	40	11,800	12,000
	20	11,100	14,200
3	40	7,600	8,400
	20	7,800	11,200
4	40	7,100	7,100
	20	4,750	9,200
	10	2,000	9,800
5	40	11,000	10,000
	20	9,400	12,000
	10	5,100	20,000
6	40	5,800	5,800
	20	1,650	5,600
7	40	10,350	10,350
	20	7,100	11,200

than that obtained with 40 mgm. (Table III). It seems that in some cases this addition increases the activity, in some other cases it counteracts only the dilution effect. The use of 40 mgm. of wet weight of tissue without addition of Ca and Al furnishes a reliable basis of comparison as long as the dry weight of the tissue is nearly the same. This is the case with normal livers, and also livers of animals fed *p*-dimethylaminoazobenzene, with or without development of liver tumors. They mean dry weight of livers from over 200 rats was 29.4 (range 26 to 36) per cent of wet weight. But with tumor tissue the dry weight varied from 14 to 24 per cent of the wet weight. Thus using 40 mgm. of wet weight the amount of tumor tissue in each vessel was only about one half of that of the normal liver tissue when compared on the dry weight basis, and the addition of Ca and Al might seem necessary. In order to avoid a possible activation by these ions, which would make a comparison with normal liver tissue difficult, the tumor tissue was used in amounts double those of normal tissue (80 mgm.). Experiments on 40 mgm. of tumor tissue with addition of Ca and Al gave very similar values to those given by 80 mgm. of tissue alone.

TABLE IV: RELATION OF INITIAL TO FINAL DRY WEIGHT OF LIVERS FROM RATS FED ON A BASIC RICE DIET WITH OR WITHOUT ADDITION OF *p*-DIMETHYLAMINOAZOBENZENE

		Initial dry weight (a) as percentage of wet weight	Final dry weight (b) as percentage of wet weight	Mean	% Decrease on shaking $\left(\frac{a-b}{a} \times 100\right)$
Control diet		26.7	16.3, 16.7, 15.4, 15.4, 12.6	15.3	42.7
		30.2	16.5, 15.7, 16.5, 14.8, 16.0	15.8	47.7
		29.7	13.9, 12.7, 13.4, 12.9, 16.0	13.7	53.9
				Mean	48.1
Control diet + *p*-DAB	A	31.3	15.1, 15.7, 15.3, 15.1	15.3	51.1
		27.4	15.3, 15.3, 15.2, 14.8	15.1	44.9
		29.4	17.4, 17.9, 17.2, 14.7, 14.5	16.2	44.9
		28.6	12.2, 12.4, 11.4, 12.5, 14.8	12.5	56.3
				Mean	49.3
Control diet + *p*-DAB	B	29.0	14.1, 14.7, 14.7, 14.1, 12.8	14.1	51.4
		28.4	15.3, 15.3, 15.5, 15.5, 15.7	15.4	45.8
		26.8	13.2, 12.7, 14.8, 14.8, 15.5	14.2	47.0
				Mean	48.1
			MEAN	48.6	

A = livers of animals without tumors.
B = normal parts of livers of animals with liver tumor.



Oxidative activity of liver homogenates and slices.

Upper 4 curves in each figure represent oxygen uptake of livers from control rats; lower 4 curves, from rats on same diet with addition of 0.06 per cent azo compound. Values marked on any one ordinate were obtained from same liver. Oxidative activity is calculated as μl. oxygen per mgm. of initial dry weight per hour for homogenates and per mgm. of final dry weight per hour for slices.

A and A_1 O---O = Succinoxidase activity of homogenates without added cytochrome c. B and B_1 ●---● = Succinoxidase activity of homogenates with excess cytochrome c added. C and C_1 ●——● = Succinoxidase activity of slices. D and D_1 O——O = Oxygen uptake of slices without addition of succinate. A, B, C, and D represent values from control rats. A_1, B_1, C_1, and D_1 represent values from rats receiving *p*-dimethylaminoazobenzene. T = Tumor in liver. Estimations of oxidative capacity were carried out on tumor-free parts of liver.

FIG. 1.—Oxidative activity of liver homogenates and slices from rats on rice and carrots with and without addition of *p*-dimethylaminoazobenzene.

FIG. 2.—Oxidative activity of liver homogenates and slices from rats on rice, 6 per cent casein, and carrots with and without addition of *p*-dimethylaminoazobenzene.

FIG. 3.—Oxidative activity of liver homogenates and slices from rats on rice and milk with and without addition of *p*-dimethylaminoazobenzene (first series).

All results of oxygen uptake are calculated on a mgm. dry weight basis. In order to compare the oxygen uptakes per unit weight of liver tissue in homogenates and in slices it was necessary to establish for the present experiment the relation of the dry weight of the slices after having been subjected to the shaking in the Warburg vessel for 1½ to 2 hours at a rate of 110 to 120 per minute (final dry weight) to the dry weight of the original liver tissue (initial dry weight). The latter was determined on tissue which had been cut into small pieces before being dried (5). It was found that the tissue loses 42.7 to 56.3 per cent (mean 48.6 per cent) of its original weight during the shaking in the Warburg apparatus (Table IV). In the literature the factor of 50 per cent is generally used for calculating the final dry weight from the initial. Since this loss is variable the calculation of the oxygen uptake on the basis of the final dry weight of the slices, as is generally

done, involves some uncertainty. The use of the final dry weight for calculating the oxygen uptake is based on the assumption that this is the weight of the tissue active in the respiration (5). This holds as long as processes bound to intact cells are measured. But succinoxidase is active also in disintegrated tissue and the initial wet weight of the slices would be a better basis for calculation. On the other hand, the necessity for weighing the tissue slices before the experiment would prolong the time-interval between the killing of the animal and the immersion of the slices in the nutrient solution and affect the viability of the cells. In the tables the oxygen uptake of the homogenates is calculated on the basis of initial, that of slices on the basis of final, dry weight.

In comparing the succinoxidase activity of homogenates with that of slices it also has to be taken into account that the homogenates generally do not show any oxygen uptake without addition of substrate; while the slices have a definite oxygen uptake. Whether the succinoxidase activity found in the slices should be corrected for autorespiration has been questioned. Rosenthal and Drabkin (15) maintain that the oxidation of a substrate added in excess might completely suppress the autorespiration and so the two values are not superimposed.

TABLE V: OXIDATIVE ACTIVITY OF LIVER HOMOGENATES FROM RATS ON CONTROL DIETS AND ON DIETS WITH ADDITION OF *p*-DIMETHYLAMINOAZOBENZENE

				μl O_2 uptake with excess of Na succinate per mgm. initial dry weight per hour					
				No cytochrome *c* added			Cytochrome *c* added		
Supplement to rice diet	No. estimations		Sex	Range	Mean	Standard error	Range	Mean	Standard error
Carrots		7	♀	15-23	19.6	1.1	29-41	31.9	1.6
"		7	♂	13-28	18.9	1.9	16-29	26.2	2.6
Carrots + *p*-DAB	A	8	♀	12-24	17.7	1.3	19-23	25.0	2.0
	B	8		13-21	16.4	1.1	21-36	27.6	1.9
6% Casein + carrots		9	♀	19-33	23.2	1.6	28-54	35.5	3.6
6% Casein + *p*-DAB	A	12	♀	14-32	21.1	1.3	17-39	29.0	2.1
	B	11		14-30	20.8	1.4	23-42	33.0	1.9
	C	1		....	3.7	..	....	6.2	..
Carrots†		10	♀	21-33	24.5	1.3	27-47	35.1	2.4
Carrots, azobenzene		9	♀	16-23	18.7	0.9	18-32	25.0	2.0
Milk†		*9	♀	26-36	28.6	1.0	30-45	36.2	1.0
		8		26-36	28.9	1.1	31-45	37.0	0.7
" †		*15	♂	11-27	18.6	1.1	19-38	24.8	1.4
		6		16-27	21.8	1.7	24-38	29.9	2.0
"		*17	♀	15-31	21.9	1.3	20-41	29.0	1.6
		11		19-31	24.5	1.3	23-41	31.0	1.9
"		*24	♂	11-32	20.3	1.2	16-47	29.1	1.6
		11		13-32	22.0	1.7	20-37	30.4	2.5
Milk + *p*-DAB Series 1	A	*18	♀	11-33	19.7	1.3	12-37	28.8	1.6
		13		19-33	21.8	1.2	21-37	28.0	1.6
	B	2		17-18	17.7	..	25-27	26.2	..
	A	*25	♂	7-32	15.7	1.2	9-40	24.4	1.7
		6		9-32	19.3	3.7	9-36	23.1	4.3
Milk + *p*-DAB Series 2	A	12	♀	19-32	24.9	1.2	25-44	34.2	1.7
	B	1		....	23.5	..	....	34.2	..
	C	1		....	2.7	..	....	6.2	..
	A	6	♂	18-27	23.5	1.3	28-46	36.3	2.6
30% Fat, 17% protein, carrots†		9	♀	8-25	17.1	2.0	19-33	27.3	1.7
30% Fat, 17% protein, carrots + *p*-DAB†	A	1	♀	....	18.6	..	....	27.8	..
	B	5		12-20	14.7	1.4	25-42	32.3	4.2
	C	5		0.5-4	2.6	0.6	0.8-28	13.0	5.5
30% Fat, 17% protein, carrots + *p*-DAB†	A	2	♂	11-21	15.8	..	16-35	25.4	..
	B	3		5-17	12.9	..	7-25	17.8	..
	C	2		3-5	3.8	..	5-7	6.1	..

With the animals receiving *p*-dimethylamincazobenzene, A = Liver of animals without tumors; B = Normal parts of livers of animals with liver tumor; C = Tumor of the liver.

* Including experiments carried out on homogenate only. Lower values are comparable with the values given in Table VI.

† With exception of groups so marked, all diets contain 2% arachis oil.

RESULTS

(*a*) *Homogenates.*—Figs. 1 to 6 show the succinoxidase activities of all rat livers estimated in slices and in homogenates. Table V summarizes the findings. Considering the values for oxygen uptake of homogenates of rats on different control rice diets without addition of *p*-dimethylaminoazobenzene there is a slight increase in the oxygen uptake with increasing protein content of the diet, both with and without addition of an excess of cytochrome *c*. This seems to point to an increasing succinic dehydrogenase content of livers with increasing protein in the diet. But in all the animals on control diets without addition of arachis oil the succinoxidase and cytochrome c content of the homogenates is higher than that with diets containing arachis oil. In the semisynthetic diet with much higher protein content, but also much higher fat content, the succinoxidase activity is the lowest. It seems that, keeping the fat values constant, the succinoxidase activity varied with the protein in the diet, but that a high fat content in the diet diminished the succinoxidase activities even of livers from rats receiving fairly high (17 per cent) protein. The tumors grew much more quickly on the fat-rich diet; the time before their appearance however was not shortened.

Eighty to 100 per cent of the rats which had received *p*-dimethylaminoazobenzene with the rice, rice and casein, or the fat-rich diet, developed tumors in the liver after 90 days. The tumors which developed in animals on rice diet were generally too small for separate estimation of the succinoxidase content. During the whole course of the experiment the succinoxidase activities of the liver homogenates of these rats were slightly lower than those of animals on the same diet without addition of the dye. The difference however was in no case statistically significant. Similarly the decrease of the succinoxidase activity in the healthy parts of the livers from animals developing tumors was not significant. But the tumor tissue itself showed always a very low oxygen uptake without addition of cytochrome *c*. On the addition of cytochrome *c* the succinoxidase activity increased and in two cases reached a value as high as found in normal liver tissues.

A curious behavior was observed in the first series of animals (50 rats receiving *p*-dimethylaminoazobenzene in the rice diet with addition of milk. The oxygen uptake in the presence of an excess of succinate without addition of cytochrome *c* dropped to a very low level 3 days after the beginning of the experiment (Fig. 3) and remained low until the 150th day. At that date the oxygen uptake rose to normal values and remained normal until 460 days. The succinoxidase activity of the homogenate with excess of cytochrome *c* was about the same during the whole course of the experiment. Thus the percentage increase of the oxygen uptake on addition of cytochrome *c* was much higher in the first 150 days. These rats did not develop hepatic lesions until the 430th day. The 2 rats killed on the 430th and 460th days respectively showed incipient cystic cholangiomas. In a second series of 30 rats this initial fall in the succinoxidase activity was not observed. Four of these animals developed tumors of the liver (but only in one case was the succinoxidase activity estimated).

No other differences could be observed between the 0_2 uptake with the added Na succinate of homogenates of livers from rats on control diets and on the same diets containing *p*-dimethylaminoazobenzene, irrespective of whether the animal developed tumors or not. Changes observed in the succinoxidase activity of liver homogenates from rats receiving 0.06 per cent azobenzene with a rice carrot diet were not significant.

(*b*) *Slices.*—The succinoxidase activities of liver slices from animals on rice, or rice and casein diets dropped on addition of *p*-dimethylaminoazobenzene. A further drop was found in normal parts of the livers from animals bearing tumors, and in tumors very low figures were obtained. In the first series of animals on the rice and milk diet, where the rats were protected considerably from tumors of the liver, the succinoxidase was not estimated in slices during the first 150 days of the experiment. After the 150 days, when the succinoxidase activity of the homogenates suddenly rose, the succinoxidase activities of the slices were within the normal range. The two rats which developed tumors had low succinoxidase activity in the slices. In the second series of rats on rice and milk diet with *p*-dimethylaminoazobenzene the succinoxidase activity of the slices was depressed.

The great discrepancy between homogenate and slices in the response of the succinoxidase activity to the feeding of *p*-dimethylaminoazobenzene could suggest that the basis of comparison might be inadequate in these cases. Since the values for the slices are calculated from the final dry weight one might assume that during the shaking in the Warburg vessel the liver tissues from animals receiving *p*-dimethylaminoazobenzene lose less weight and consequently the values for the oxygen consumption divided by abnormally high values for the dry weight will give very low figures for succinoxidase activity. The histological evidence of cirrhosis in these livers would give support to this assumption. To test this, initial wet weights and final dry weights of livers from control rats and rats receiving *p*-dimethylaminoazobenzene were measured. No differences in the different livers were found (Table IV).

The values of the oxygen uptake of the slices from

livers of rats on the control diets divided by the ratios of initial to final dry weight give exactly the values obtained with homogenates without the addition of cytochrome *c*. This seems to suggest that the effective succinoxidase activity of the intact tissue is equal to the succinoxidase activity of homogenates to which no cytochrome *c* is added; which would also imply that, in rats under the described dietary conditions, not all the succindehydrogenase is active *in vivo*. In slices of livers from rats receiving *p*-dimethylaminoazobenzene the oxygen uptake is lower than that calculated from the oxygen uptake of homogenate and the ratio of initial to final dry weight.

DISCUSSION

The absolute values for the succinoxidase activity for the various organs even in the same species of animal given in the literature vary widely; *e.g.* for rat liver slices Roskelley and his co-workers (16) give Q suc from 18.5 to 23.1 and Rosenthal and Drabkin (15) Q suc 46.7 ± 1.51 as a mean. For liver homogenates Elliott and Greig (5) give the values 66μ 0_2 and Schneider and Potter (18) 87.7 (from 76.8 to 101μ 0_2) for 1 mgm. of dry tissue for 1 hour. The relative order of the succinoxidase content of different tissues is generally found the same. The experiments presented here and other experiments on the influence

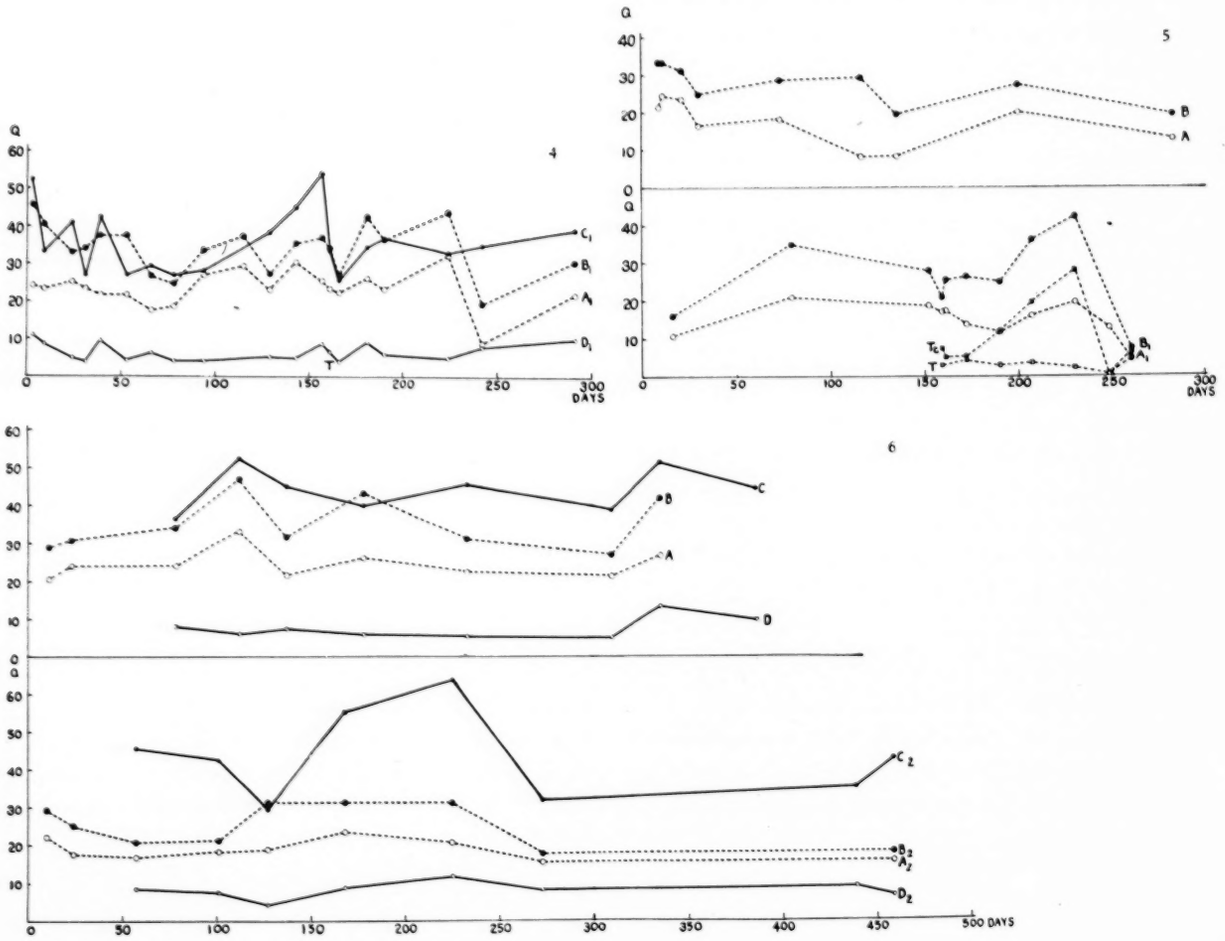


FIG. 4.—Oxidative activity of liver homogenates and slices from rats on mice and milk with addition of *p*-dimethylaminoazobenzene (second series).

FIG. 5.—Oxidative activity of liver homogenates from rats on diet containing 17 per cent protein and 30 per cent fat with and without addition of *p*-dimethylaminoazobenzene. T □---□ = Succinoxidase activity of tumor tissue homogenates without addition of cytochrome c. T_c ●---● = Succinoxidase activity of tumor tissue homogenates with excess cytochrome c added. The values (A_1 and B_1) above T and T_c are obtained in tumor-free parts of livers.

FIG. 6.—Oxidative activity of liver homogenates and slices from rats on rice and carrots without arachis oil, and same diet with addition of 0.06 per cent azobenzene in 2 per cent arachis oil. A and A_2 O---O = Succinoxidase activity of homogenates without added cytochrome c. B and B_2 ●---● = Succinoxidase activity of homogenates with excess cytochrome c added. C and C_2 ●——● = Succinoxidase activity of slices. D and D_2 O——O = Oxygen uptake of slices without addition of succinate. A, B, C, and D represent values from control rats. A_2, B_2, C_2, and D_2 represent values from rats receiving azobenzene.

TABLE VI: OXIDATIVE ACTIVITY OF LIVER SLICES FROM RATS ON CONTROL DIETS AND ON DIETS WITH ADDITION OF *p*-DIMETHYLAMINOAZOBENZENE

Supplement to rice diet	No. estimations	Sex	μl. O_2 uptake with excess of Na succinate per mgm. final dry weight per hour: Found Range	Found Mean	Found Σ	Corrected for autogenous O_2 uptake Range	Corrected for autogenous O_2 uptake Mean	Corrected for autogenous O_2 uptake Σ
Carrots	7	♀	34-74	51.5	5.5	26-60	42.4	4.7
"	7	♂	29-62	47.8	4.5	23-47	37.7	3.4
Carrots + *p*-DAB	A 8	♀	21-49	34.3	2.9	14-42	26.5	2.9
	B 8		15-33	23.8	2.2	10-26	16.9	2.0
6% Casein + carrots	9	♀	39-55	47.8	2.1	30-49	40.2	2.6
6% Casein + carrots + *p*-DAB	A 12	♀	23-45	32.4	2.3	17-36	25.5	2.0
	B 11		21-36	30.1	1.5	17-29	22.7	1.2
	C 1		....	13.5	..	....	5.9	..
Carrots*	10	♀	37-52	44.3	2.0	28-46	36.6	1.8
Carrots, azobenzene	9	♀	30-64	43.3	4.1	24-52	35.0	3.6
Milk*	8	♀	53-78	63.4	1.1	35-69	54.0	1.3
" *	6	♂	43-85	67.8	5.2	32-73	58.2	5.1
Milk	11	♀	33-70	50.3	4.2	27-62	41.5	4.0
"	11	♂	29-77	51.1	4.0	23-63	39.9	3.6
Milk + *p*-DAB Series 1	A 13	♀	35-73	51.6	3.4	25-58	41.4	3.3
	B 2		28-24	26.0	..	22-17	19.3	..
" "	A 6	♂	36-63	47.7	3.7	26-53	37.5	3.9
Milk + *p*-DAB Series 2	A 12	♀	27-45	35.4	2.0	22-40	29.3	1.8
	B 1		....	33.5	..	....	26.9	..
	C 1		....	12.4	..	....	....	..
" "	A 7		27-54	36.9	4.3	23-45	29.6	3.6

With the animals receiving *p*-dimethylaminoazobenzene, A = livers of animals without tumors; B = normal parts of livers of animals with liver tumor; C = tumor of the liver.

* With the exception of the groups so marked, all diets contain 2% arachis oil.

of different diets carried out in this Institute (Elson, L. A., in press) show that the succinoxidase content of liver can be very much depressed by dietary means. Workers in America report generally higher succinoxidase values for rats than do workers in England. Perhaps the standard diet of the American rats is better. In experiments with diets containing high percentages of fat low succinoxidase values in the liver of rats were found; values which were well within the range for hepatic tumors from rats. The figures for succinoxidase for homogenates of hepatomas produced by *p*-dimethylaminoazobenzene is given by Schneider and Potter (18) as Q suc 25.0 0_2. This figure is about a third of their values for normal livers but differs only by about 20 per cent from the values for normal livers as found here. On the other hand, not every hepatic tumor produced by *p*-dimethylaminoazobenzene has a low succinoxidase activity when measured as homogenate with an excess of cytochrome *c*; two tumor homogenate showed high values. The low oxygen uptake without added cytochrome *c* might indicate a low cytochrome *c* content of this tissue.

The phenomenon, that the succinoxidase activity of liver from rats receiving *p*-dimethylaminoazobenzene is lower than in the control when estimated in slices but about the same when estimated in homogenates opens several theoretical possibilities:

(a) It would seem that characteristic differences between the enzymatic behavior of livers from rats on a control diet and livers from rats developing tumors on the same diet with *p*-dimethylaminoazobenzene are connected with the intact cells. The slight differences in activity found with homogenates may be due to some intact cells still present. Enzymic activity is in many cases connected with intact cell structures. Yudkin (20) showed that the activity of glucose dehydrogenase of *Bact. coli* and lactic dehydrogenase of the *Micrococcus lysodeikticus* is linked with the structure of the cells. In the experiments presented here, however, the activity in the intact cells is lower than in the disintegrated cells. Since the ratio of initial to final dry weight in the livers which developed tumors is not different from that obtained with normal tissue, and since it is not likely that the permeability of the cell membranes to Na succinate is changed, a possible explanation of this phenomenon could be a changed mutual accessibility of the parts of the sucinic oxidase system in the intact cell. A varying accessibility of enzyme to its substrate was first suggested by Claude Bernard (2) to explain the simultaneous presence of both

glycogen and diastatic enzyme in the liver of the hibernating frog, without formation of larger amounts of glucose.

(b) The low activity of liver from animals fed *p*-dimethylaminoazobenzene may also be the result of one part of the enzymatic system being blocked by the carcinogenic substance or by a metabolite. Substances connected with the metabolism of *p*-dimethylaminoazobenzene are known to inhibit the succinoxidase activity of the liver. If such substances are present in the liver cells they might suppress the succinoxidase activity of the slices. On disintegration of the cells and dilution of the cell content the concentration of the inhibitory substance might become too low to be effective. It might be significant that in the groups of animals in which, by addition of fresh milk to the diet the formation of hepatic tumors was largely prevented, no depression of the succinoxidase activity in liver slices was found with the exception of 2 animals that had incipient cholangiomas.

SUMMARY

1. The succinoxidase activity of rat liver slices and homogenates was studied during the course of development of hepatic tumors due to feeding of *p*-dimethylaminoazobenzene. The diets of the animals consisted of rice and carrot, rice, casein and carrot, rice and milk, or a semisynthetic diet containing a high percentage of fat.

2. The succinoxidase activity of the homogenates of livers from the rats not receiving *p*-dimethylaminoazobenzene varied slightly with the diet; it was lowest with the high-fat diet and highest with the rice diet containing 6 per cent casein, where the percentage of fat was lowest.

3. After the addition of Na succinate the 0_2 uptake of homogenates of liver, with and without excess of cytochrome *c*, from rats receiving *p*-dimethylaminoazobenzene was only slightly lower than the control irrespective of whether the individual animal had developed a hepatic tumor or not. The tumors themselves showed generally a low succinoxidase activity.

4. Slices of liver from animals subsequently developing tumors on *p*-dimethylaminoazobenzene had lower succinoxidase activity than the controls. The succinoxidase activity was further depressed on the development of tumors and was very low in tumor tissue.

5. In one series of rats where the addition of milk largely prevented the development of hepatic tumors the succinoxidase activity of liver slices did not differ from that in rats on the control diet.

6. The addition of azobenzene to a rice-carrot diet had no effect on the succinoxidase activity of the homogenates or slices of livers.

7. The discrepancy between the values for succinoxidase found when using homogenates, or slices of the same liver from animals fed *p*-dimethylaminoazobenzene might be explained by a different accessibility of the parts of the enzymic system or by assuming an intracellular inhibition in these livers.

ACKNOWLEDGMENT

I wish to express my gratitude to the Sir Halley Stewart Trust for a fellowship held during this work. Also, my thanks are due to The British Empire Cancer Campaign, The Anna Fuller Fund and The Jane Coffin Childs Memorial Fund for Medical Research for grants made toward the support of the work carried out in The Chester Beatty Research Institute. I also wish to thank Miss P. Hales and Miss M. I. Houchin for their assistance.

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The Production of Tumors in the Livers of Rats Fed *m'*-Methyl-*p*-Dimethylaminoazobenzene*

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(Received for publication October 25, 1946)

Miller and Baumann (3) and Giese, Miller and Baumann (1) studied the carcinogenicity of various methyl derivatives of dimethylaminoazobenzene and found that the *m'*-methyl-*p*-dimethylaminoazobenzene was the most potent of the azo dyes for the production of liver tumors in rats, being even more active than *p*-dimethylaminoazobenzene itself. As we were interested in studying conditions of rapid carcinogenesis, *m'*-methyl-*p*-dimethylaminoazobenzene was selected as the carcinogenic agent.

In most of the early studies on the production of liver tumors, the investigators fed the dye continuously to the rats until the tumors appeared. Kinosita (2) mentioned in one of his reports that if animals were fed *p*-dimethylaminoazobenzene in the rice-carrot diet for only 50 days, and then returned to a normal diet without the dye, some of the rats would develop tumors in 150 days. Sugiura and Rhoads (9) fed *p*-dimethylaminoazobenzene to rats for 30, 45, 60, and 85 days in the rice-carrot diet, and then withdrew the dye from the diet, continuing the animals on the rice-carrot diet alone. Only those animals which received the dye for 60 and 85 days later developed tumors. Reimann, Stimson, and Medes (8) fed rats *p*-dimethylaminoazobenzene in a rice diet supplemented with fresh vegetables for 75 days and then returned them to the stock diet. One hundred and thirty days later they found that 5 out of 15 animals had developed tumors. Giese, Miller and Baumann (1) fed the *m'*-methyl-*p*-dimethylaminoazobenzene at a level of 0.056 per cent in a semi-synthetic diet for 2½ months and then continued the animals on the semi-synthetic diet alone. Two months later there was a 100 per cent incidence of liver tumors.

In the studies reported here, the animals were fed the azo dye for different lengths of time in an effort to determine the minimum possible exposure to the carcinogenic agent necessary for subsequent tumor formation. In addition, some information was obtained on the role of diet in the development of tumors following the initial period of dye feeding. In the early studies on the production of liver tumors with the azo dyes both the Japanese workers and the investigators in this country fed the dye to rats in a diet consisting of rice and carrots. The use of this diet, however, carried with it a relatively high mortality, as a rule. Miller, Miner, Rusch, and Baumann (4) and Miner, Miller, Baumann, and Rusch (5) in careful studies on the relation of diet to hepatic tumor formation found that a semi-synthetic diet low in protein and riboflavin but more than adequate in respect to other members of the vitamin B complex resulted in a very high incidence of tumors when the azo dye was incorporated in it, and at the same time, provided a diet adequate for maintenance of the animals. They found that increasing the riboflavin content of such a diet could prevent tumor formation completely. With the use of more crude diets, increase in the protein content of the diet reduced considerably the incidence of tumor formation. In the experiments reported below, the rats were fed the *m'*-methyl-*p*-dimethylaminoazobenzene in the semi-synthetic diet as described by Miller, Miner, Rusch, and Baumann (4), but following the initial period of dye-feeding some of the animals were placed on the regular stock diet and the subsequent course of tumor development was followed.

METHODS

A total of 91 adult male rats of the Sprague-Dawley strain weighing between 230 to 320 gm. at the start of the experiment were used. All of the rats were placed at first on the semi-synthetic diet of Miller, Miner, Rusch and Baumann (4), consisting of crude casein, 120 gm.; salts, 40 gm.; corn oil, 50 gm.; rice bran concentrate, 20 gm.; glucose, 770 gm.; and riboflavin, 0.5 mgm. per kgm. of diet. In addition each rat was given 1 drop of halibut liver oil monthly. Into this diet was incorporated 0.05 per cent of *m'*-methyl-

* Aided by grants from The Donner Foundation and The Jane Coffin Childs Memorial Fund for Medical Research.

** Smith, Kline, and French Fellow in Pharmacology.

p-dimethylaminoazobenzene by dissolving it by means of heat in the corn oil. The dye was synthesized according to the method described by Giese, Miller and Baumann (1). After the animals were on this diet for approximately 1 month, they began to show signs of riboflavin deficiency. The rice bran concentrate was increased to 30 gm. and the riboflavin to 0.75 mgm./kgm. of diet, after which all signs of riboflavin deficiency disappeared.

At various intervals after beginning the dye feeding, the exact times of which are stated below, a group of animals were removed from this dietary regime. Half of such a group was then placed on fox chow (Purina) and the other half was maintained on the semi-synthetic diet described above, but without the dye. The animals were then observed until tumors developed, or for approximately 190 days, at which time all remaining animals were killed. The abdomen was palpated for the presence of a tumor and the first appearance of such was noted. Usually animals were not killed until the tumors had grown to considerable size, but in several cases rats died as the result of the tumor growth. At various intervals in the experiment representative rats were killed for examination of the liver both grossly and microscopically.

All the rats were allowed food and water *ad libitum*. Food intake was not measured but body weight was recorded weekly to determine the progress of the animals.

RESULTS

Eleven rats received *m'*-methyl-*p*-dimethylaminoazobenzene in the semi-synthetic diet for 39 days. Following this 5 of the rats were continued on the semi-synthetic diet without the dye and 6 were given fox chow. These rats were followed for an additional 146 days, at which time they were sacrificed and their livers examined for the presence of tumors. In none of these animals was there any evidence of tumor formation, either grossly or microscopically.

Fourteen rats received the *m'*-methyl dye in the semi-synthetic diet for 46 days. Then 7 rats were given fox chow and 7 were continued on the semi-synthetic diet without the dye. These rats were followed for an additional 151 days, at which time they were sacrificed. In this group likewise there was no evidence of any tumor formation.

Thirty rats received the *m'*-methyl dye for 69 days. Following this the rats were divided into two groups; 15 rats were continued on the semi-synthetic diet without the dye, and 15 rats were fed fox chow for the duration of the experiment. The first palpable tumor appeared in a rat receiving the semi-synthetic diet on the 24th day following discontinuance of the dye feeding. This was slow-growing and did not become of any considerable size until 90 days after discontinuance of the dye. In the group receiving fox chow, the first tumors appeared on the 42nd and 55th days after discontinuance of dye feeding. On the 81st day following discontinuance of dye feeding, of the 15 rats in the fox chow group, 10 already showed evidence of tumor formation, either by the definite presence of a palpable mass in the abdomen or demonstrated by autopsy. In the group receiving the semi-synthetic diet alone, 6 of 15 showed evidence of tumor formation by the 81st day. On the 115th day after discontinuance of the dye feeding, all remaining animals that had not died or been sacrificed in the meantime were killed and examined for tumor formation. Final count in the two groups showed the following tumor incidence: 14 of 15 animals on fox chow had tumors, an incidence of 93 per cent, whereas 12 of 15 animals on the semi-synthetic diet alone had tumors, an incidence of 80 per cent. The data on the relation of the duration of dye feeding to the incidence of tumor formation are summarized in Table I.

TABLE I: RELATION OF THE DURATION OF DYE FEEDING, AND OF THE SUBSEQUENT DIET TO THE PRODUCTION OF LIVER TUMORS IN RATS FED *m'*-METHYL-*p*-DIMETHYLAMINOAZOBENZENE

No. of rats	No. of days on exper. diet plus azo dye	No. of days on exper. diet alone	No. of days on fox chow alone	No. of rats with tumors 81 days after dye stopped	No. of rats with tumors, final incidence
6	39		146	0	0
5	39	146		0	0
7	46		151	0	0
7	46	151		0	0
15	69		81-115	10	14
15	69	81-115		6	12

In the course of the autopsy of these rats the lungs were examined grossly for any suspicious nodules, and if such were present, microscopic examination of the nodules were carried out. Since some of the animals died as a result of their tumors, only those animals on which adequate autopsy was performed are reported here. Of the 7 rats receiving the semi-synthetic diet alone, the lungs of which were examined carefully following death, none showed any gross evidence of the presence of tumor nodules of any kind. Of the 9 rats on the fox chow diet, which were similarly autopsied, 5 showed definite gross and microscopic evidence of the presence of tumor metastases varying in size from 1 mm. to as large as 8 mm. These were in all cases identified as coming from the liver tumors. Although no microscopic examination was made of the lungs of animals showing no nodules grossly, still it is possible to conclude from the data presented here that at least the extent of tumor metastases was significantly greater in rats whose diet following the period of dye ingestion was fox chow.

The 30 rats that ingested the azo dye for 69 days showed the following weight changes. During the course of the dye feeding, the average gain per rat for the entire group over the 69 day period was 7 gms. For the 15 rats on fox chow, the average gain per rat after 81 days was 104 gm. The rats placed on the semi-synthetic diet alone for the corresponding period showed an average increase of 45 gms. per rat. Incorporation of the azo dye into the semi-synthetic diet prevented almost completely any weight increase which the animals would have had on the semi-synthetic diet alone. Although food intake was not measured, the much greater increase in weight of the animals that were continued on fox chow indicates a food consumption greater than that in animals maintained on the semi-synthetic diet alone.

Microscopic examination of the tumors appearing in the liver in the course of these experiments showed that in general the types of tumors produced and their development following feeding with *m'*-methyl-*p*-dimethylaminoazobenzene were very similar to those produced in rats by *p*-dimethylaminoazobenzene, as described by Orr (7) and Opie (6). Like these authors we found that the tumors could be classified as bile duct carcinoma (cholangioma), bile duct cystadenoma and liver cell carcinoma, and that in many instances more than one type of tumor appeared in the same animal.

DISCUSSION

These studies have shown that in the production of tumors in rat livers by feeding *m'*-methyl-*p*-dimethylaminoazobenzene in a semi-synthetic diet low in protein and riboflavin, the duration of feeding the dye will determine the rate of tumor formation. Although it is not necessary to administer the carcinogenic agent continually, nevertheless the animals must be exposed to its effects for a minimum length of time in order that tumors may develop. In animals that ingested the dye for only 39 and 45 days tumors never developed, whereas animals exposed to the dye for 69 days later developed a high incidence of tumors. It would seem that during this period the neoplastic focus has developed which can now proceed independently of the exciting agent to the formation of tumor.

In liver carcinogenesis with the azo dyes, the simultaneous feeding of an inadequate diet, particularly in respect to protein and riboflavin, has been found necessary for the production of a high incidence of tumors. These experiments show, however, that once the neoplastic focus has arisen, the maintenance of this low-protein, low-riboflavin diet is not essential for the further growth and development of the tumors. The animals receiving the azo dye in the semi-synthetic diet for 69 days and then receiving the fox chow diet showed a high incidence of tumor formation. In comparing these animals with those maintained on the semi-synthetic diet alone, the final incidence of tumor formation on the fox chow diet is slightly greater (14 of 15 compared with 12 of 15) but not significantly so because of the relatively small number of animals used in the series. However, the animals receiving the fox chow diet after their initial exposure to the azo dye showed an earlier onset of tumor formation as indicated by the greater tumor incidence at 81 days as compared with the animals on the semi-synthetic diet. Furthermore the tumors of the group fed fox chow showed a significantly greater development of lung metastases.

Tannenbaum (10) has clearly shown for many different types of mouse tumors, both spontaneous and induced, that restriction of caloric intake decreases the incidence and delays the formation of tumors. The greater weight gain of the animals fed fox chow indicates a higher food consumption for these animals. Their increased caloric intake undoubtedly accounts in great part for the earlier development of hepatic tumors and their more widespread metastates as compared with the animals on the semi-synthetic diet, whose food intake was lower. Evidence for the role of the other dietary factors, such as changes in protein and riboflavin level, on tumor development following the minimum period of exposure to the azo dye cannot be adduced from the data presented here because of the co-existing variations in caloric intake, which of themselves influence tumor development.

SUMMARY

1. Rats fed *m'*-methyl-*p*-dimethylaminoazobenzene in a semi-synthetic diet low in protein and riboflavin for 39 and 46 days, then followed for 150 days without the dye, developed no liver tumors whether the diet during the dye-free period was semi-synthetic or fox chow.

2. Rats which were fed the azo dye in the semi-synthetic diet for 69 days, and then placed on dye-free diet, developed a high incidence of tumors. Approximately 93 per cent of the animals placed on fox chow following the dye-feeding period had tumors, whereas 80 per cent of those maintained on the semi-synthetic diet developed tumors. The tumors of the animals receiving fox chow appeared earlier and were more malignant as evidenced by the greater incidence of lung metastases.

3. The histopathology of liver tumors produced by *m'*-methyl-*p*-dimethylaminoazobenzene was found to be similar to that of tumors produced by the parent dye, *p*-dimethylaminoazobenzene, namely liver cell carcinoma, bile duct cystadenoma, and cholangioma.

ACKNOWLEDGMENT

I am grateful to Dr. H. S. N. Greene, of the Department of Pathology, who reviewed some of the tumor slides with me.

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p-Dimethylaminoazobenzene Carcinogenesis with Purified Diets Varying in Content of Cysteine, Cystine, Liver Extract, Protein, Riboflavin, and Other Factors*

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(Received for publication October 12, 1946)

INTRODUCTION

The experiments to be described in this paper were begun with a number of objectives in view: first, to study tumor production by *p*-dimethylaminoazobenzene in rats upon diets having a well-defined composition from a nutritional standpoint; secondly, to determine under such well-defined nutritional conditions the effects of riboflavin and of liver extract, which had been previously reported to reduce tumor incidence when given as supplements to a polished rice diet; and thirdly, to follow tumor development throughout the entire life span of a population rather than to limit the observations, as had been usually done, to determination of tumor incidence at the expiration of some arbitrarily chosen period of time.

As the experiments proceeded, tests of the effects of substances other than riboflavin and liver extract upon tumor development were carried out; the effects of cysteine, cystine, *p*-aminobenzoic acid, pantothenic acid, choline, inositol, lipocaic, and succinic acid will be reported in this paper. The effects of biotin and egg albumin will be reported in subsequent communications.

Since these experiments were begun in January, 1941, reports upon the effects of a number of these agents have appeared from other laboratories. In spite of a certain degree of overlapping with such previously published data, the present experiments are presented in some detail because they give a complete record of tumor development with each combination of agents used.

METHODS

The rats used in these experiments were obtained from a local breeder and were descendants of the Wistar strain. Their weights at the beginning of treatment ranged from 75 to 120 gm. but few animals of less than 90 gm. were used, and the greater number weighed 100 to 110 gm.

The carcinogen, *p*-dimethylaminoazobenzene, obtained from Eastman Kodak Company, was administered in the rats' food. The first lot of dye obtained was recrystallized, but this procedure was omitted as unnecessary with subsequent lots. Instead of dissolving the dye in olive oil as others have done, we used cottonseed oil (Wesson Oil), dissolving 30 gm. in 970 gm. of oil with the aid of moderate heat. Thirty grams of this solution were thoroughly mixed with 970 gm. of diets 1 to 10, 15 to 24, 30, 34, and 35. One gram of the final mixture thus contained 0.9 mgm. of the dye, a concentration one-half again as great as has been used by most workers. In diets 25 to 29 the dye content was reduced to 0.6 mgm. per gram, only 20 grams of the solution being added to 980 grams of the diet, and with diets 41 and 42 a similar reduction was achieved by addition of 30 gm. of a 2 per cent solution of the dye to 970 gm. of the diet. The carcinogen content was lowered with the hope of reducing the mortality rate in the first weeks of each experiment.

The animals were kept in groups of five per cage, in an air-conditioned room at a temperature of 76° to 80° F. No measurement of the individual food intake could be made. In order to prevent accumulation of old food, the food cups were allowed to become empty at weekly intervals, but at all other times they were kept filled. With the three exceptions noted below (*see* Results), the carcinogen was administered continuously until the rats died or were killed.

Six different basal diets have been used. One consisted solely of finely ground polished rice. The composition of the other five is given in Table I. Basal diets 1, 3, and 4 contained 20 per cent casein and differed only in riboflavin content. Basal 3 was low, basal 1 intermediate, and basal 4 relatively high in riboflavin. Basal 5 differed from basal 3 in containing only 10 per cent casein, one half as much thiamin, two thirds as much vitamin B_6, and no choline. Basal 2 was low in riboflavin, and contained no casein and no choline.

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* Presented at the 37th Annual Meeting of the American Association for Cancer Research at Atlantic City, New Jersey, March 11, 1946.

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TABLE I

Basal Diets

	No. 1	No. 2	No. 3	No. 4	No. 5
Casein, vitamin-free, Labco	200.00	0.0	200.00	200.00	100.00
Crisco or Primex	50.00	50.00	50.00	50.00	50.00
McCollum's Salt Mixture No. 185* (modified)	40.00	40.00	40.00	40.00	40.00
Agar†	20.00	20.00	20.00	20.00	20.00
Carotene	0.01	0.01	0.01	0.01	0.01
Vitamin D Concentrate in cottonseed oil (400,000 U. per gm.)	0.005	0.005	0.005	0.005	0.005
Thiamin	0.005	0.005	0.005	0.005	0.0025
Riboflavin	0.005	0.001	0.0015	0.008	0.0015
Vitamin B_6	0.003	0.003	0.003	0.003	0.002
Nicotinic acid	0.01	0.005	0.01	0.01	0.01
α-Tocopherol‡	0.01	0.01	0.01	0.01	0.01
Calcium pantothenate	0.0056	0.0056	0.0056	0.0056	0.0056
Choline chloride	1.0	0.0	1.0	1.0	0.0
Cerelose, refined§	689.0	0.0	689.0	689.0	790.0
Ground polished rice	0.0	890.0	0.0	0.0	0.0

* This was modified by the addition of 0.4 gm. of KI, 0.72 gm. of $CuSO_4$, and 1.0 gm. of $MnCl_2$ to 5916.6 gm. of salts mixed according to McCollum's formula.

† After February, 1942, agar had to be omitted from the diets, and pieces of filter paper were fed to the rats. Some of the animals of diets 1, 10, 17, 19, 20, 24, 25, and 26 were subjected to this change. Animals receiving diets 27 to 42 never had agar. An additional 2% of carbohydrate was substituted for agar.

‡ In the later experiments this was supplied as distilled natural tocopherols.

§ In February, 1943, it became necessary to replace cerelose by starch. Some of the rats on diets 30 to 35, inclusive, were subjected to this change.

The composition of the diets made from the various basal diets is given in Table II.

TABLE II

Diets

1. 4850 gm. 1st Basal
   150 " Carcinogen solution
2. 4704.5 gm. 1st Basal
   150 " Carcinogen solution
   145.5 " Liver Extract, Lilly
3. 4845 gm. 1st Basal
   150 " Carcinogen solution
   24.5 cc. Liver Extract Solution, Purified, Lilly*
5. 4845 gm. 1st Basal
   150 " Carcinogen solution
   4.85 " *p*-Aminobenzoic acid
8. 4850 gm. 1st Basal
   150 " Carcinogen solution
   165 mgm. Pantothenic acid
10. 4800 gm. 1st Basal
   150 " Carcinogen solution
   50 " *l*-Cysteine hydrochloride
17. 4850 gm. 1st Basal
   150 " Carcinogen solution
   125 mgm. Riboflavin
19. 4842.5 gm. 1st Basal
   150 " Carcinogen solution
   7.5 " Choline hydrochloride
20. 4845 gm. 1st Basal
   150 " Carcinogen solution
   5 " Inositol
9. 4850 gm. Ground polished rice
   150 " Carcinogen solution
15. 4850 gm. 2d Basal
   150 " Carcinogen solution
16. 4704.5 gm. 2d Basal
   150 " Carcinogen solution
   145.5 " Liver Extract, Lilly
27. 4850 gm. 2d Basal
   100 " Carcinogen solution
   50 " *l*-Cysteine hydrochloride
28. 4895 gm. 2d Basal
   100 " Carcinogen solution
   5 " *l*-Cysteine hydrochloride
29. 4850 gm. 2d Basal
   100 " Carcinogen solution
   50 " *l*-Cystine
30. 4100 gm. 2d Basal
   150 " Carcinogen solution
   750 " Liver Extract, Lilly
21. 4850 gm. 3d Basal
   150 " Carcinogen solution
22. 4800 gm. 3d Basal
   150 " Carcinogen solution
   50 " *l*-Cysteine hydrochloride
35. 4600 gm. 3d Basal
   150 " Carcinogen solution
   250 " Lipocaic
23. 4850 gm. 4th Basal
   150 " Carcinogen solution
24. 4800 gm. 4th Basal
   150 " Carcinogen solution
   50 " *l*-Cysteine hydrochloride
34. 4825 gm. 4th Basal
   150 " Carcinogen solution
   25 " Succinic acid
41. 4800 gm. 4th Basal
   150 " Carcinogen solution (2% Dye)
   50 " *l*-Cystine
42. 4800 gm. 4th Basal
   150 " Carcinogen solution (2% Dye)
   50 " *l*-Cysteine hydrochloride
25. 4900 gm. 5th Basal
   100 " Carcinogen solution
26. 4754 gm. 5th Basal
   100 " Carcinogen solution
   146 " Liver Extract, Lilly

* Equivalent to twice the amount of fresh liver represented by the crude liver extract used in diet No. 2.

At first, for each group of rats fed a diet containing the carcinogen, a control group of rats was fed the same diet without the oily solution of carcinogen. This procedure was soon abandoned since it did not yield enough information to justify its continuance. The livers of these control animals were ultimately found to be normal. As would be expected, none of our basal diets permitted maximal growth, and addition of the carcinogen resulted in much additional retardation of growth.

As a means of reducing the mortality rate as much as possible, the rats that received the rice diet or modification of the second basal diet were also given 10 gm. of carrot each twice weekly. This procedure nullifies the advantages offered by an exact synthetic diet, and the necessity for such supplementation to a diet renders the use of that diet undesirable. In the case of diets 16 and 30 this supplementation was discontinued as unnecessary after several weeks.

All animals were weighed twice weekly for several months, and thereafter only once weekly except in case of diets on which the rats gained little or lost weight. After three months' administration of the dye, palpation of the abdomen was performed weekly. This method is not entirely satisfactory, for the readiness with which a tumor may be palpated depends not merely upon its size, but largely upon its location, *i.e.* whether it lies entirely within the lobe and causes a smooth enlargement, or is superficial and forms a projecting nodule, and upon its situation with respect to the costal margin. A tumor arising in the cephalad part of the liver may become quite large before a definite diagnosis can be made, although the liver margin is obviously lower than normal, whereas a tumor more accessible to palpation may be discovered when only a few millimeters in diameter. The development of cirrhosis with formation of large nodules of regenerated liver cells also renders diagnosis difficult. Hence, tumors that appeared in rats given a diet productive of severe cirrhosis sometimes reached a larger size before being diagnosed than would have been the case had the diet offered protection against cirrhosis. After sufficient experience had been gained, uncertainty as to the presence of a tumor was usually dispelled within a week or two. Rapid growth of a mass and the degree of firmness and the contour of the masses aided in distinguishing between carcinoma and cirrhosis. Development of cirrhosis preceded by several weeks the appearance of tumors. Occasionally, formation of large numbers of cysts led us to a mistaken diagnosis of cancer.

TABLE III

Diet No.	Date begun 1941	Total used	Effective Total	Tumors	Minimal latent period, days	50% Tumor incidence, days	In Tumor Rats: Cirrhosis: Much %	Cirrhosis: Slight %	Cirrhosis: Focal %	Fibrosis %
1	Jan. 14	90	62	55	104	212	0	6	31	72
1*	July 29	40	31	28						
2	Jan. 14	30	26	24	112	184	0	0	8	52
3	Jan. 15	30	28	26	139	205	4	0	24	48
5	"	30	23	22	132	183	5	5	14	77
8	Mar. 15	30	17	17	124	174	0	12	41	76
9	Apr. 25	44	19	15	90	163	73	27	0	47
10	Jan. 15	30	28	22	119	296	0	6	21	48
10*	Sept. 11	30	19	12						
15	May 19	55	34	30	87	139	83	13	3	43
16	"	40	28	22	143	401	0	9	18	73
17	Aug. 21	30	27	23	96	173	4	0	31	65
19	Sept. 16	30	23	21	134	250	10	33	33	50
20	"	30	17	15	126	235	20	40	20	73
21	Sept. 11	30	20	15	72	153	100	0	0	33
22	Sept. 22	30	26	25	113	152	52	36	8	48
23	Sept. 12	30	21	18	131	227	6	33	39	45
24	Sept. 29	30	18	9	198	408	11	11	22	44
25	Dec. 16	50	32	29	120	162	86	14	0	31
26	"	25	23	13	162	455	0	31	15	54
	1942									
27	Mar. 16	25	23	22	114	176	90	5	5	72
28	"	25	20	19	121	142	100	0	0	50
29	"	25	22	21	127	169	81	19	0	43
30	Apr. 21	25	20	17	106	266	24	18	30	41
34	Apr. 28	25	20	19	176†	232	10	25	35	10
35	May 8	25	23	20	155†	213	0	40	35	50
	1944									
41	July 24	25	22	11	148	561	0	25	25	34
42	"	34	20	9	222	never	0	9	36	9

* Repeat.
† Latent period too long, unforeseen circumstances having prevented palpation sooner than five months.

In calculating tumor incidence, the number of rats on any given diet living at the time the first tumor was diagnosed and which were subsequently examined at necropsy has been used. This number is referred to in Table III as the effective total. The point of 50 per cent tumor incidence is the time at which half of the effective total of animals developed tumors. The minimum latent period of Table III gives the earliest time at which we were sure a tumor was present. In most instances the rats were killed soon after the tumor was detected. Some rats died with small tumors that could not have been palpated. Several rats that had palpable tumors were not included in the figures since they died and were eaten by cagemates before confirmation of the diagnosis by inspection of the liver could be accomplished. Several presumably tumor-free rats were excluded from the data for the same reason. The livers of all animals included in the tables have been examined microscopically.

Each point on the experimental curves (Figs. 18 to 24) represents the detection of a tumor in one or more animals. The exact number of animals affected at any point can be determined from the effective total in Table III and the tumor percentage shown on the ordinate of the curve under consideration. The death of a tumor-free animal is indicated by a short line perpendicular to the curve.

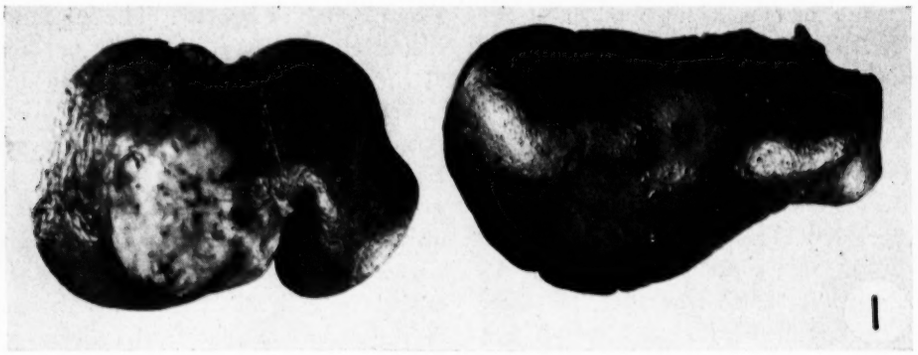
The procedure, adopted by some investigators, of killing all rats in a group at a given time makes it possible to get an accurate figure of the incidence of tumor formation at that time, but is subject to the disadvantage of not permitting observations at later intervals, and hence may predispose to the drawing of erroneous conclusions. Our method, while less accurate at whatever time interval is selected for termination of the experiment in the other method, gives a more accurate overall picture. For example, if we had killed all our animals at the end of 6 months, we should not have learned that with continuous administration of the carcinogen, animals that receive a tumor-retarding diet may ultimately have a tumor incidence nearly as high as that of the controls. (For example, compare curves of diets 15 and 16, Fig. 21.) Frequent laparotomies of experimental animals are not feasible, and it would be impractical to run experiments on such a scale as to permit the killing of groups of 20 or 25 rats at weekly intervals in order to ascertain the tumor incidence in experiments that may run for more than a year.

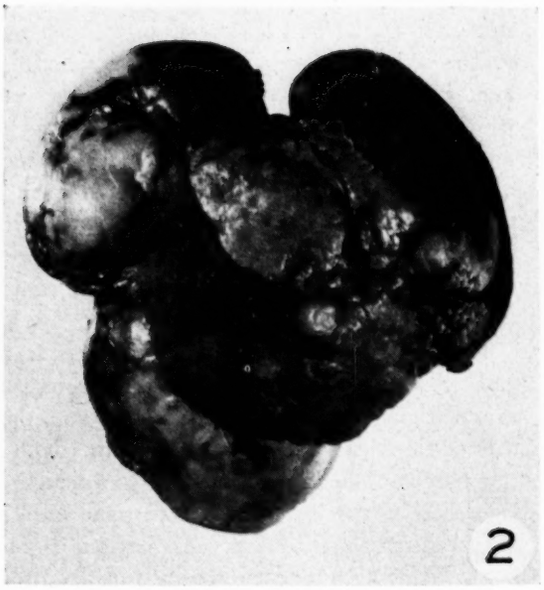
RESULTS

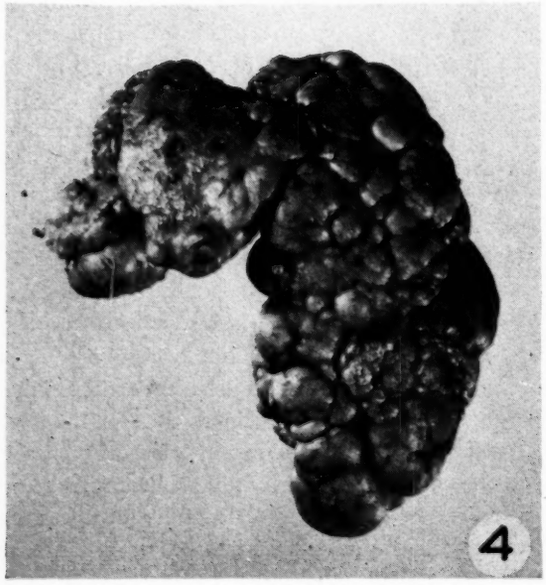
Histological observations.—Nodular cirrhosis developed regularly and reached an advanced stage in animals receiving the polished rice diet and diets made with basal 2, 3, and 5. Exceptions are diets 16, 26, and 30, to which liver extract had been added, and diet 35, which contained lipocaic. The incidence of cirrhosis on diet 22 was somewhat less than on diet 21 (see Table III, which shows the incidence and degree of cirrhosis among tumor-bearing rats on all diets). It will be noted that the diets that produce cirrhosis contain little riboflavin and that, except for basal 3, they provide little protein and no choline. Animals that received diets made from basal diets 1 and 4 never developed extensive cirrhosis, and only a few developed so much as a slight, grossly recognizable cirrhosis. Although development of slight, and usually focal, microscopic cirrhosis was not uncommon, many rats did not develop even a microscopic cirrhosis (*i.e.* no increase in collagen was demonstrable with Mallory's aniline blue stain). By focal microscopic cirrhosis we mean that there were foci in which single lobules or only a few lobules were surrounded by delicate bands of collagenous tissue. These bands ramified outward between the adjacent lobules to a variable extent. In such foci, bile duct proliferation was usually slight. It was obvious that diffuse, nodular cirrhosis would result from growth and confluence of such foci.

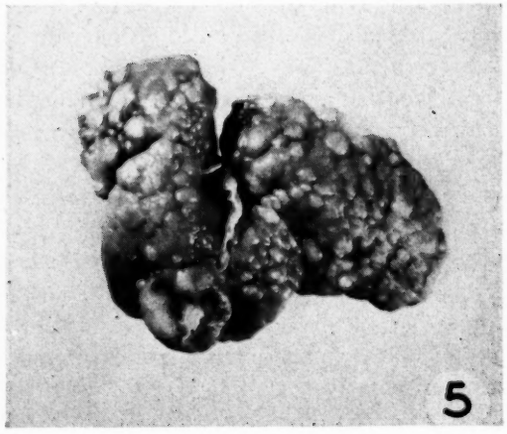
In an otherwise grossly normal liver, we not infrequently observed foci of fibrosis that were usually located in the right half of the median lobe and the anterior half of the right lateral lobe. At times both halves of the median lobe were involved, and occasionally the right half of the left lobe showed some fibrosis. With diffuse cirrhosis this sharp localization of fibrosis was not evident. Although fibrosis at times was so extensive as to involve an entire lobe, as a rule the lesion was focal (Fig. 7), but the foci were frequently confluent, with persisting islands of liver cells (Fig. 8.) Opie (27) has called this lesion cholangiofibrosis and has described it so well that no further description is necessary. We wish to mention its occurrence, since some workers have apparently considered it a manifestation of cirrhosis. Although it was found regularly in severely cirrhotic livers, it also occurred to some degree in otherwise normal livers, and frequently, but not invariably, in livers that showed only slight focal cirrhosis.

The predominance of localization of fibrosis in the right half of the median lobe and anterior half of the right lateral lobe is of interest, as is the fact that in livers which were not grossly cirrhotic, tumors tended to show the same distribution. Examples are shown by Figs. 1, 2, and 3. The number of livers containing tumors in these two locations only, even though tumors were multiple, was more than twice as great as the number in which tumors were found in other lobes alone or in other lobes as well as the ones mentioned. When cirrhosis-producing diets were administered, this peculiar localization of tumors was not observed. Furthermore, with cirrhosis-producing diets, tumors were









FIGS. 1 TO 5

usually multiple, whereas with the other diets tumors were frequently single. Even with cirrhosis-producing diets, Opie (27) found more tumors on the right side of the liver.

The relation of cirrhosis to tumor development is worthy of comment. In the early work on *p*-dimethylaminoazobenzene carcinogenesis, cirrhosis occurred with such regularity that Sugiura and Rhoads (31) contrasted the action of this dye with that of *o*-aminoazotoluene, remarking that with the former the usual sequence is, first the development of cirrhosis with subsequent appearance of tumors, whereas with the latter compound cirrhosis generally does not develop. Yet Maruya (13) remarked that cirrhosis is not necessary for tumor production, and the data of the group at the University of Wisconsin (14, 18) and of Opie (27) confirm this observation. Our data also show that tumors develop readily in the absence of cirrhosis. While it is true that tumors appear earlier in rats fed a cirrhosis-producing diet, this does not necessarily mean that cirrhosis in itself promotes carcinogenesis, for Kline (10) observed that addition of *p*-aminobenzoic acid to the diet caused great reduction of cirrhosis without changing the incidence of liver cancer.

Inasmuch as the pathogenesis of *p*-dimethylaminoazobenzene carcinogenesis has been adequately discussed by others, notably by Opie (27) and Edwards and White (5), it is unnecessary to present an account here. However, a few general remarks will not be amiss. We have obtained a variety of tumors. Among the malignant tumors there was a wide variety of types ranging from well differentiated adenocarcinoma through mixtures of adenocarcinoma and malignant hepatoma (a very common type), to pure malignant hepatoma, and to a completely undifferentiated type of tumor composed of masses of small basophilic cells. These are illustrated in Figs. 9 to 17.

We cannot say how many of these tumors are derived from liver cells and how many from bile ducts. We are convinced that most of them arise from liver cells, although some evidently arise from bile duct epithelium. The mere formation of glandular structures in a liver tumor is not proof of its origin from bile ducts. Gomori (6) has stated recently that hepatomas are lipase positive and cholangiomas are lipase negative. Until *p*-dimethylaminoazobenzene tumors are studied by a method such as Gomori's, the question as to percentage of each type of tumor will remain a matter of personal opinion.

Histologic changes other than tumor formation have been described by Opie (27), Edwards and White (5, 34), György, Poling, and Goldblatt (7), Antopol and Unna (2), and Orr (28), so that here, too, a detailed discussion is unnecessary. After several weeks of administration of the dye a change became apparent in the size and staining capacity of liver cells, and was more conspicuous in animals that were killed than in those that died spontaneously. Some cells were paler and slightly or considerably larger than the others, and had coarsely granular cytoplasm. Other cells were small, rather dark and basophilic, and had finely granular or homogeneous cytoplasm; mitoses in these cells were sometimes fairly numerous. The basophilic cells appeared to originate about the portal spaces. Cells of either of these types sometimes extended from one edge of a lobule toward, to, or beyond a central vein. Transition from small to large, or darkly to lightly stained cells was abrupt (Fig. 6). This change was seen even after many months of treatment, and in tumor-free as well as tumor-bearing livers. In cirrhotic livers the change was less striking, for although the lobules varied much from one another, the individual lobules were fairly uniform. Similar changes have been mentioned by György, Poling, and Goldblatt (7), and by Opie (27).

Not infrequently, cells of malignant, as well as of benign tumors contained large fat globules. This is shown in Fig. 15. In the case of malignant tumors the cells so affected were apparently relatively less malignant than the others, but mitoses have been seen in fat-containing cells. Occasionally fat storage in tumors was much more prominent than in the non-neoplastic liver cells.

There was great difficulty in distinguishing between neoplasia and regeneration in livers of some of the

DESCRIPTION OF FIGURES 1 TO 5

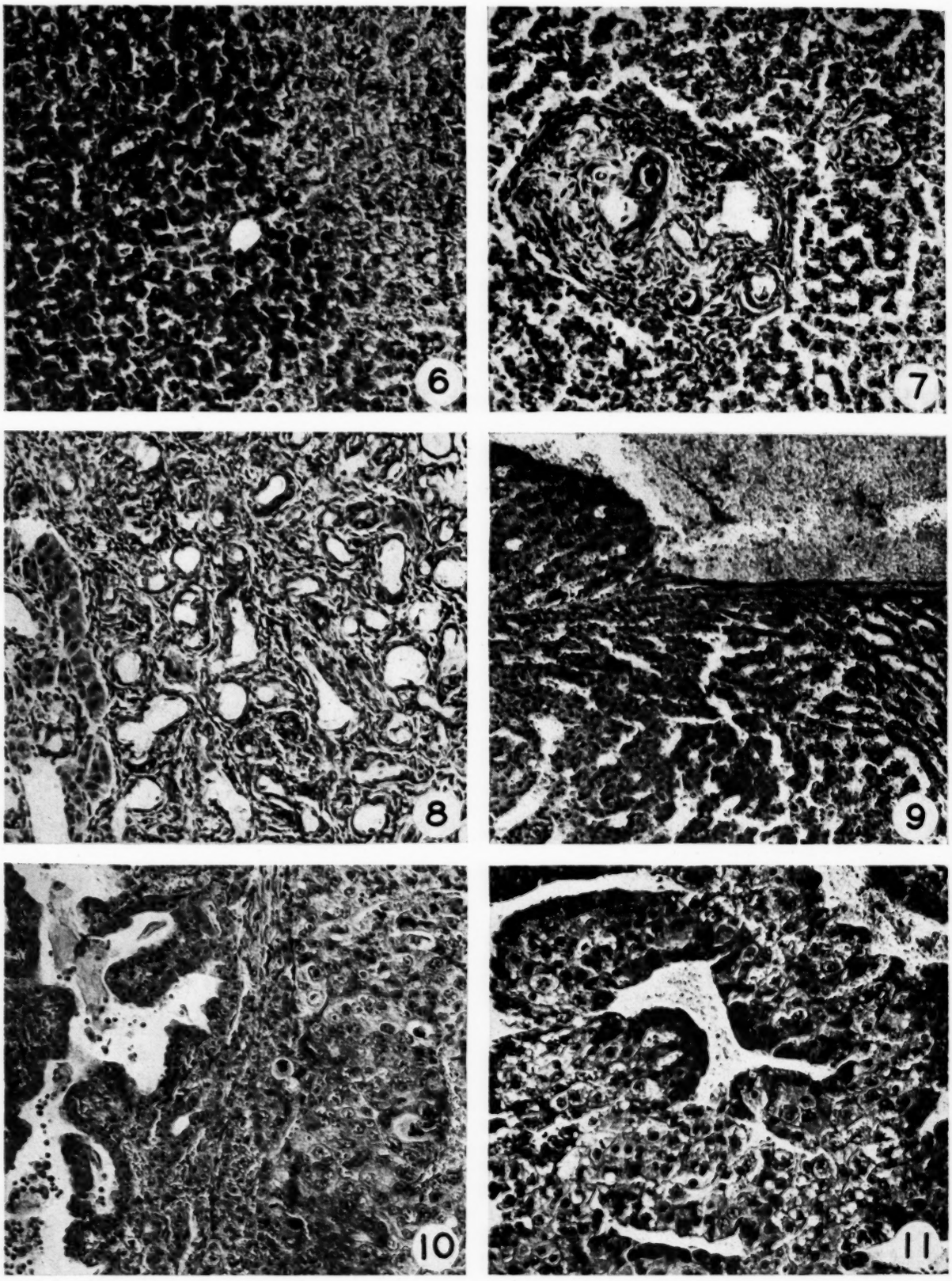
FIG. 1.—Liver of rat 954, killed after 205 days on diet 26. There is some scarring lateral to tumor in right half of median lobe, but remaining tissue is smooth. Mag. × 1.2.

FIG. 2.—Liver of rat 67, killed after 194 days on diet 1. Median lobe is shrunken and fibrotic, and left lobe is scarred. Tumors are present in right and left lobes. Mag. × 1.2.

FIG. 3.—Liver of rat 22, killed after 203 days on diet 1. A large tumor arises from right half of median lobe. There is no cirrhosis. Mag. × 1.2.

FIG. 4.—Liver of rat 746, killed after 140 days on diet 21. There is cirrhosis with much regeneration of liver tissue. Right half of median lobe is fibrotic. There is no tumor. Mag. × 1.2.

FIG. 5.—Liver of rat 393, killed after 86 days on diet 9. The liver is cirrhotic, and in right half of median lobe there is indented tumor 1 cm. in diameter. Mag. × 1.2.



FIGS. 6 TO 11

animals. Nodules which were not unquestionably neoplastic were classed as non-neoplastic. This may result in our having a lower incidence of tumors than we should, but error in this direction is preferable to error in the other.

With three exceptions, administration of the dye was continued until the animals died or were killed. Two of the three rats (Nos. 337 and 338, diet 8) received the dye for 259 days, and then were given our colony diet. One animal was found to have liver cancer 71 days later, the other lived 222 days before tumor was detected. The third rat (No. 91, diet 2) received the dye for 379 days, and after 69 days on the colony diet was found to have liver cancer.

The data for all diets are summarized in Table III. The effects of variation of riboflavin and casein content of the basal diets, and of addition of liver extract, of cysteine, and of cystine, are recorded graphically in Figs. 18 to 24.

Effect of variation in riboflavin and casein content of diet.—With 20 per cent casein in the basal diet, four levels of riboflavin were employed: 1.5, 5, 8, and 26.5 mgm. per kgm. of basal diet. The times at which 50 per cent of the animals developed tumors on diets 21, 1, 23, and 17 were 153, 212, 227, and 173 days respectively (*See* Fig. 18).

The addition of 1 mgm. of riboflavin per kgm., with a supplement otherwise adequate, to a polished rice diet produced no delay in tumor development as compared with that on polished rice alone. The times for 50 per cent tumor development in diets 15 (with supplement) and 9 (polished rice alone) were, respectively, 139 and 163 days. (Fig. 19).

The effects of 10 and 20 per cent casein were compared at a single low riboflavin level of 1.5 mgm. per kgm. basal diet. The times at which 50 per cent of animals developed tumors on diets 25 (10 per cent casein) and 21 (20 per cent casein) were 162 and 153 days, respectively (Fig. 20); the effect of the added casein under these circumstances, in which the low riboflavin content apparently acted as a limiting factor, was negligible. Also, at this riboflavin level, the time for tumor development with 10 or 20 per cent casein was nearly the same as with the basal diet 2, polished rice containing 1 mgm. per kgm. of riboflavin (diet 15). Thus, addition of casein causes no delay in tumor development unless riboflavin is also increased to an adequate level which, in our experience, appears to be about 5 to 8 mgm. per kgm. of basal diet.

These data confirm the observation of the Memorial Hospital group (8) that, with adequate casein, increase in the riboflavin intake to an optimal level reduces tumor incidence or delays tumor development. The apparently smaller protection afforded by supraoptimal riboflavin intake remains unexplained.

Effect of liver extracts.—Two types of liver extract were tested: Liver Extract, Lilly (earlier known as Liver Extract No. 343), and secondly, Liver Extract Solution Purified, Lilly, a concentrate containing a higher ratio of anti-pernicious anemia principle per unit weight of solids than Liver Extract, Lilly.

On a diet of polished rice with vitamin supplements adequate in factors other than riboflavin (1 mgm. per kgm.), 3 per cent liver extract delayed tumor development; the times at which 50 per cent of animals developed tumors were 139 days (control, diet 15) and 401 days (3 per cent liver extract, diet 16). *See* Fig. 21. This confirms and extends the observations of the Memorial Hospital group (32) as to the protective effect of liver extract.

When the content of Liver Extract, Lilly, was increased to 15 per cent (diet 30), there was less protection (50 per cent tumors at 266 days). This lower protection at higher liver dosage is believed to be due, at least in part, to the biotin content of the liver and will be discussed further in a later paper.

When tested against a control diet containing 10 per cent casein and 1.5 mgm. per kgm. of riboflavin, 3 per cent of liver extract gave a degree of delay in tumor development comparable to that obtained upon the polished rice basal diet, the times at which 50 per cent of the animals developed tumors being 162 days (control, diet 25) and 455 days (3 per cent Liver Extract Lilly, diet 26). *See* Fig. 22.

When tested against a more adequate diet containing 20 per cent casein and 5 mgm. per kgm. of riboflavin, 3 per cent of liver extract gave no delay, or even perhaps an acceleration, in tumor development, the times at

DESCRIPTION OF FIGURES 6 TO 11

FIG. 6.—There is abrupt transition from small cells with dark cytoplasm to larger cells with pale cytoplasm. Mag. × 122.

FIG. 7.—A focus of cholangiofibrosis. Mag. × 122.

FIG. 8.—An example of more extensive cholangiofibrosis. Mag. × 122.

FIG. 9.—Invasion of a vein by an otherwise apparently benign hepatoma. Mag. × 122.

FIG. 10.—A common type of malignant tumor incorporating glandular and solid trabecular arrangement of cells. Mag. × 122.

FIG. 11.—Malignant tumor of hepatoma type with cells arranged in broad cords. Mag. × 122.

which 50 per cent of animals developed tumors being 212 days (control, diet 1) and 184 days (3 per cent liver, diet 2). *See* Fig. 22. It will be noted that both the relative and absolute delay produced by 3 per cent liver extract were less under these conditions than on the 10 per cent casein-low riboflavin diet.

Liver Extract Purified, Lilly, used in an amount equivalent to twice the amount of original liver represented by the 3 per cent Liver Extract, Lilly, likewise gave no protection on the 20 per cent casein—5 mgm. per kgm. riboflavin diet.

The variation in effect of liver extract, dependent upon the concentration used and the nature of the basal diet, illustrates the need for caution in interpretation and comparison of results obtained by different investigators, and the desirability of controlling the components of the diet as closely as possible.

Effect of cysteine and cystine.—Cysteine (as *l*-cysteine hydrochloride) was tested at 0.1 per cent and 1 per cent against the low-riboflavin—polished-rice diet (basal diet 2) and at 1 per cent against diets containing 20 per cent casein and varying amounts of riboflavin.

Upon the low-riboflavin—polished-rice basal diet, 0.1 per cent cysteine gave no protection, but 1 per cent gave a slight delay, the times at which 50 per cent of animals developed tumors being 139 days (control diet 15), 142 days (0.1 per cent cysteine, diet 28), and 176 days (1 per cent cysteine, diet 27). *See* Fig. 23.

Upon the same test diet, 1 per cent cystine also gave a slight delay (169 days, diet 29, compared to 139 days, diet 15). *See* Fig. 23.

With a 20 per cent casein diet containing only 1.5 mgm. of riboflavin per kgm., addition of 1 per cent of cysteine had no appreciable effect upon tumor development (50 per cent tumors at 153 days with diet 21 and at 152 days with diet 22). With larger amounts of riboflavin in the diet there was consistent lengthening of the time for 50 per cent tumor development: at 5 mgm. per kgm. the interval was 296 days (diet 10) as compared to 212 days (diet 1); and at 8 mgm. per kgm. the interval was 408 days (diet 24) compared to 227 days (diet 23). *See* Fig. 24.

Since cysteine had caused such striking retardation of carcinogenesis under proper conditions, and since the statements in the literature concerning the effect of cystine (7, 14, 21, 33, 34) were contradictory, and in the main opposed to our findings with cysteine, it seemed desirable to compare the effect of the addition of these two amino acids to our fourth basal diet. Accordingly, diets 41 (1 per cent cystine) and 42 (1 per cent cysteine) were prepared. At this time the concentration of *p*-dimethylaminoazobenzene in cottonseed oil solution used in preparation of the diets was reduced from 3 per cent to 2 per cent, resulting in a reduction of dye content of the diet from 0.9 mgm. per gm. to 0.6 mgm. per gm. This reduction of carcinogen content resulted in an even greater retardation of tumor development than was noted with diet 24, but the point of significance is that both cystine and cysteine had the same effect in retarding carcinogenesis. *See* Fig. 24.

Effects of p-aminobenzoic acid, pantothenic acid, choline, inositol, succinic acid, and lipocaic.—Under the conditions of test, none of these substances affected significantly the rate of tumor development.

The first four substances were tested against a control diet containing 20 per cent casein and 5 mgm. riboflavin per kgm. (basal diet 1). The times at which 50 per cent of the animals developed tumors were: control (diet 1), 212 days; 0.1 per cent *p*-aminobenzoic acid (diet 5), 183 days; 55 mgm. pantothenic acid per kgm. of diet (diet 8), 174 days; 0.15 per cent choline (diet 19), 250 days; 0.1 per cent inositol (diet 20), 235 days.

Succinic acid was tested against a control diet containing 20 per cent casein and 8 mgm. riboflavin per kgm. The times at which 50 per cent of animals developed tumors were 227 days for the control (diet 23), and 232 days for the 0.5 per cent succinic acid diet (diet 34).

A preparation of lipocaic (supplied by Mr. G. B. Walden, Lot H7254) was added at a level of 5 per cent to a diet containing 200 gm. of casein and 1.5 mgm. of riboflavin per kgm. (basal 3). As compared with diet 21 (basal 3 plus the carcinogen), the lipocaic diet showed striking retardation of carcinogenesis, but the curve of tumor development was nearly identical with

DESCRIPTION OF FIGURES 12 TO 17

FIG. 12.—A benign hepatoma, the cells and nuclei of which vary greatly in size. The trabeculae also vary in width. Mag. × 122.

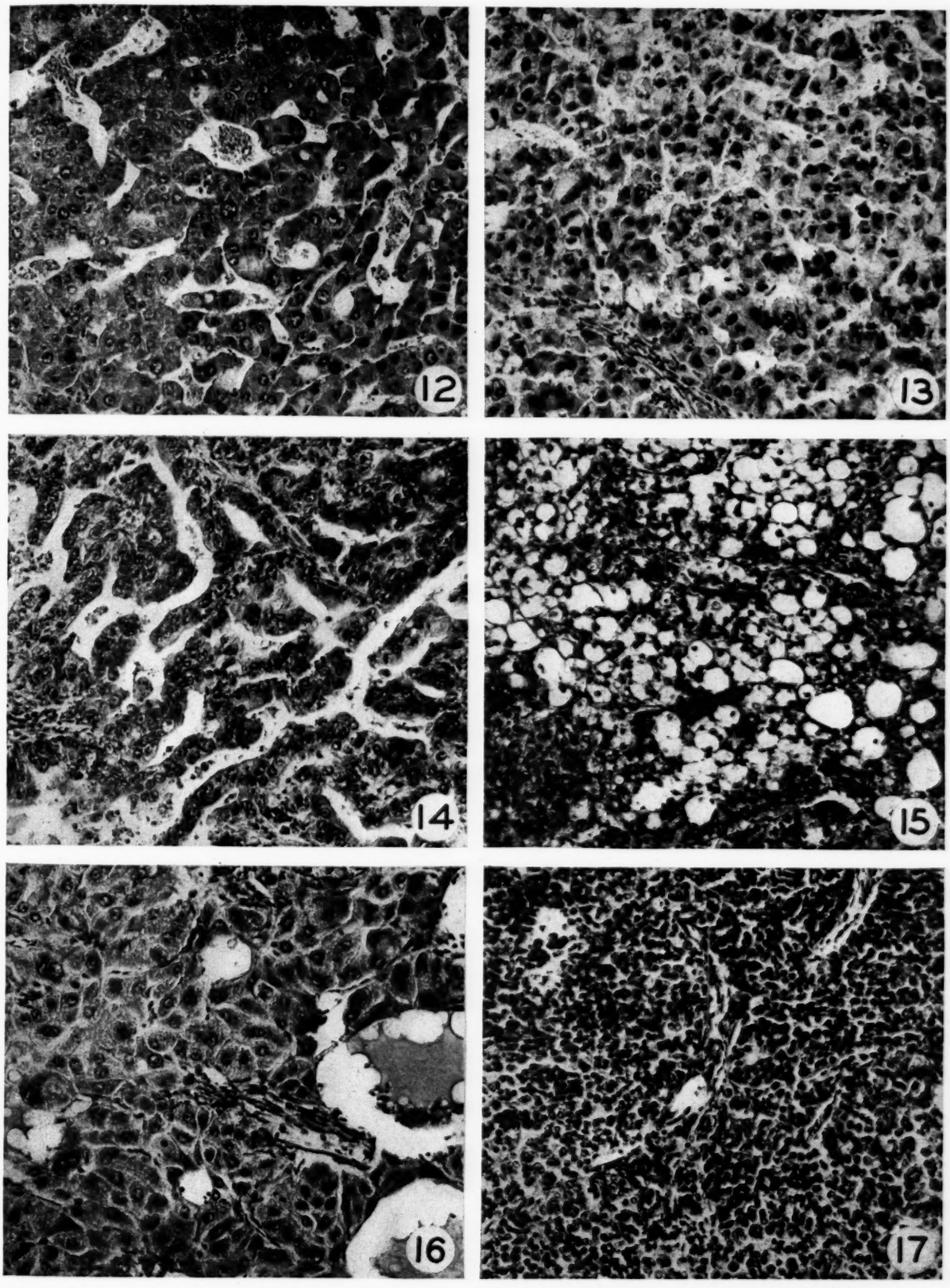
FIG. 13.—A malignant hepatoma with many mitoses. The cell cords are narrow. Mag. × 122.

FIG. 14.—An adenocarcinoma. Mag. × 122.

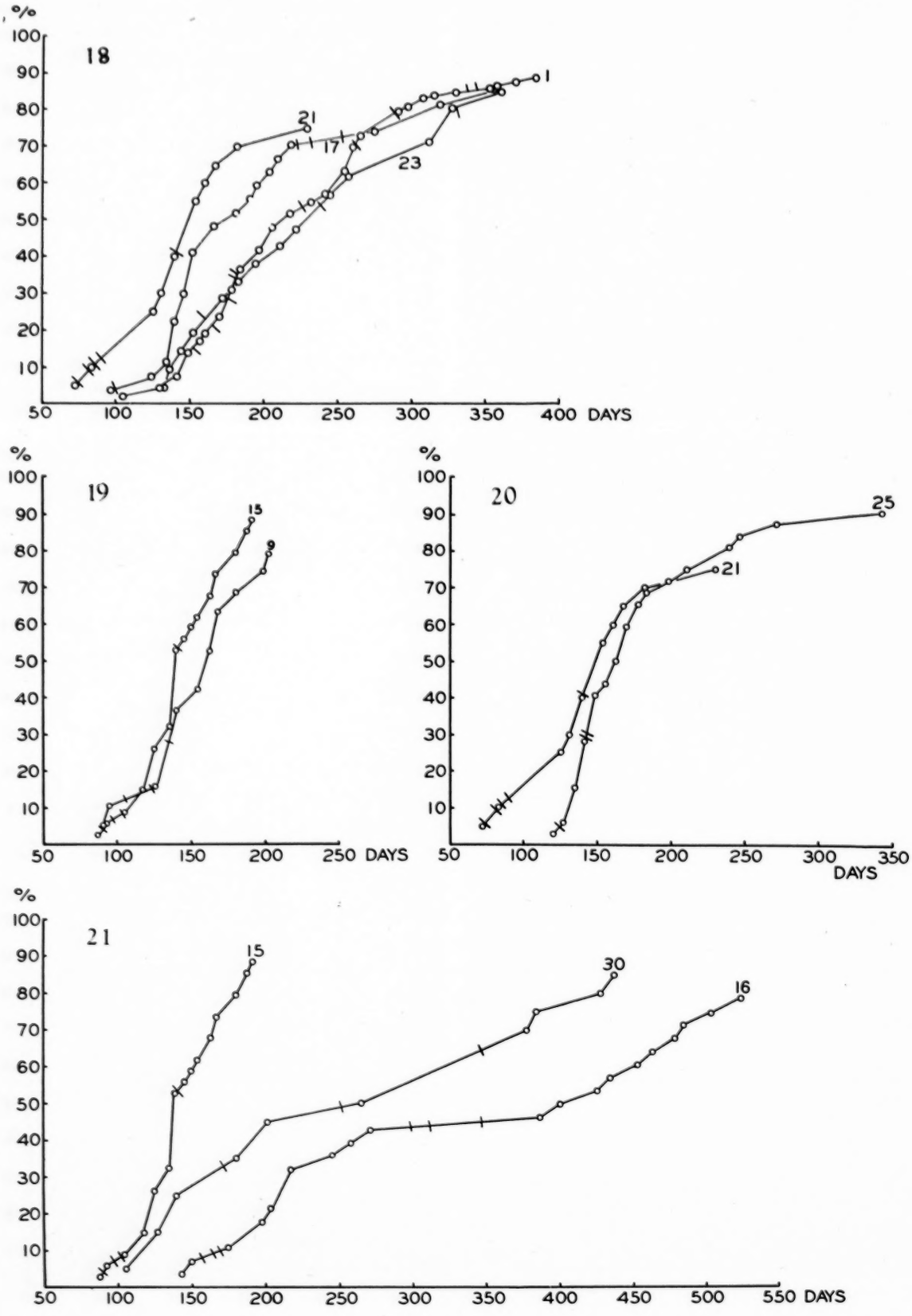
FIG. 15.—Many tumor cells contain large fat globules. Mag. × 122.

FIG. 16.—A tumor composed of cells suggestive of squamous epithelium, but without intercellular bridges. Mag. × 122.

FIG. 17.—A tumor composed of small undifferentiated epithelial cells. Mag. × 122.



FIGS. 12 TO 17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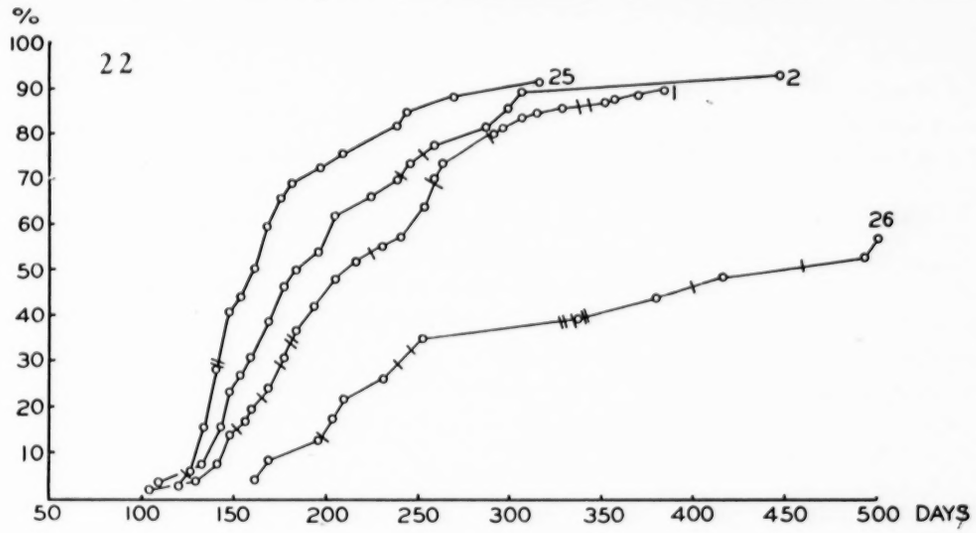

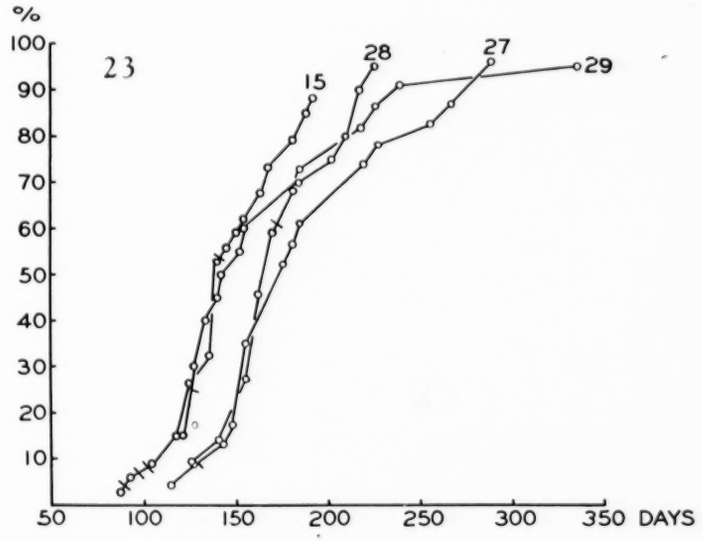

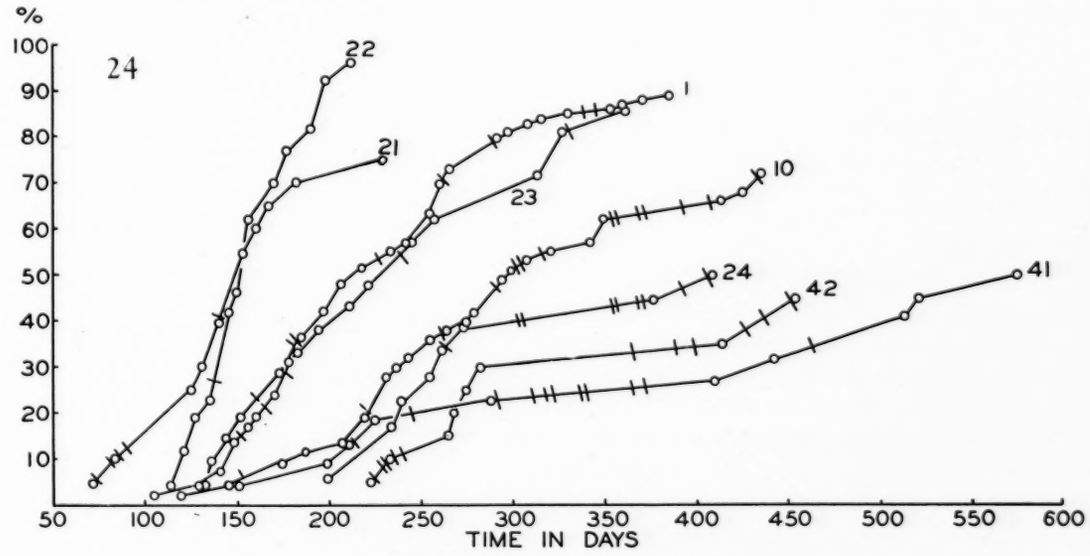

DESCRIPTION OF FIGURES 18 TO 24

FIGS. 18, 19, and 20.—The effect of variation in riboflavin and casein content of the diet upon carcinogenesis.

FIGS. 21 and 22.—The effect of liver extract upon carcinogenesis.

FIGS. 23 and 24.—The effect of cysteine and of cystine upon carcinogenesis.

FIGS. 18 to 24 show cumulative tumor percentage on various diets. The composition of the diets is given in Tables 2 and 3. Death of a tumor-free animal is indicated by a short line perpendicular to the curve.

that of diets 1 and 23. Since the sample of lipocaic contained 139 μgm. of riboflavin per gm., the actual riboflavin content of diet 35 was 8.5 μgm. per gm., a level comparable to that in diet 23. It is thus evident that lipocaic in itself did not affect carcinogenesis.

DISCUSSION

Perusal of the literature on *p*-dimethylaminoazobenzene carcinogenesis leaves the impression that some authors regard substances such as yeast and liver as offering complete protection against tumor development, whereas long-term experiments such as those here reported indicate that such protection is only partial, and that tumor development is retarded rather than entirely prevented. It has been shown (9) that administration of the compound for fifty days suffices for ultimate carcinogenesis, and it is possible that if we had stopped the administration at that time the effect of tumor retardation would have been more striking.

A variety of agents has been reported to retard tumor development. These include liver (14, 19, 23, 25), liver extracts (14, 30, 32), kidney (20), yeast (14, 24, 29, 30, 31), ether extract of yeast (29), alcohol extract of yeast (32), rice bran oil (9), ether extract of rice bran (29), rye (12), wheat (1), millet (22), riboflavin and casein together (2, 8, 14, 30), egg albumin (11, 14), and hydrogenated coconut oil (15, 16).

Tumor development is reported to be accelerated by splenectomy, by blockage of the reticuloendothelial system, by addition of cholesterol or lanolin to the diet, and by simultaneous administration of phosphorus or arsenic (9). A procarcinogenic action has been reported for biotin (3, 4), pyridoxin (17, 18), inositol (14), rice (26), and fat (26).

Conflicting observations have been made upon the effect of cystine. György, Poling, and Goldblatt (7) found that simultaneous administration of cystine and choline offered definite, but not regular protection against liver injury by *p*-dimethylaminoazobenzene, but White and Edwards (34) using an almost identical diet, could not confirm these observations. In fact, addition of cystine and choline to the diet raised the tumor incidence from 60 per cent to 90 per cent, and addition of methionine had the same effect. Mori (21) found that the addition of 0.1 per cent cystine to the diet had no effect on tumor development, but White and Edwards (33) found that tumor development on a diet low in cystine was notably retarded as compared with tumor development on the same diet supplemented by the addition of cystine at the level of 0.5 per cent. Miller, Miner, Rusch and Baumann (14) also found that a diet containing 0.5 per cent cystine favored tumor development.

SUMMARY

1. In most of these experiments the carcinogen was administered at a level of 0.9 mgm. per gm. of diet, although in some the level was reduced to 0.6 mgm. per gm. Administration was terminated only with death of the animals.

2. Retardation of carcinogenesis was effected by incorporation in the diet of the following substances: 1 per cent of cysteine; 1 per cent of cystine; 5 to 8 mgm. of riboflavin per kgm. of a 20 per cent casein diet; and 3 per cent of liver extract added to a diet favorable to early carcinogenesis.

3. Three per cent of liver extract added to a diet that in itself protected against carcinogenesis did not offer further protection.

4. Fifteen per cent of liver extract in the diet offered much less protection than did 3 per cent of liver extract. This effect is attributed to biotin.

5. The following substances had no appreciable effect upon carcinogenesis under the conditions of our experiment: choline; inositol; *p*-aminobenzoic acid; pantothenic acid; lipocaic; and succinic acid.

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The Effect of Biotin upon *p*-Dimethylaminoazobenzene Carcinogenesis*

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(Received for publication October 12, 1946)

In a previous report, we (2) have shown that although addition of liver extract at a level of 3 per cent to a diet favorable to production of hepatic carcinoma by *p*-dimethylaminoazobenzene resulted in great retardation of tumor development, addition of the extract at a level of 15 per cent afforded appreciably less protection. This effect was attributed to biotin, since the 15 per cent liver extract diet contained approximately 0.2 μgm. of biotin per gram. (This estimate is based upon assay of another lot of liver extract because the lot used in the 15 per cent diet was exhausted.) With a daily food consumption of 10 gm. per rat, this would provide 2 μgm. per day, an amount found by du Vigneaud and his associates (1) adequate to accelerate tumor development on a protective diet. In order to ascertain whether or not our interpretation was valid, another experiment was set up.

METHODS

In general, our procedures were identical with those employed in the earlier experiments. However, the concentration of the carcinogen in the diets was reduced from 0.9 mgm. per gm. to 0.6 mgm. per gm. with the hope of reducing the mortality rate. Four diets were set up as shown by Table I. The composition of the basal diet is given in our earlier paper (2). In these experiments agar had to be omitted from the basal diet, and in lieu thereof, pieces of filter paper were fed to the rats twice weekly. In preparing the diet, the agar was replaced with an equal weight of carbohydrate. As before, 10 gm. of carrot were given to each rat twice weekly as long as seemed necessary. It was possible to discontinue this supplementation earlier with the diets that contained liver extract. It should be observed that diets 37 and 38 contained biotin at the level of 0.3 μgm. per gm., presumably a slightly higher level than was present in our 15 per cent liver extract diet. Except for the differences already mentioned, diets 15R and 16R were identical with our original diets 15 and 16.

TABLE I

Diet 15R		
4850	gm.	2nd basal diet
150	gm.	carcinogen solution (2% in cottonseed oil)
Diet 16R		
4700	gm.	2nd basal diet
150	gm.	Liver Extract, Lilly
150	gm.	carcinogen solution (2% in cottonseed oil)
Diet 37		
4850	gm.	2nd basal diet
0.0015	gm.	biotin*
150	gm.	carcinogen solution (2% in cottonseed oil)
Diet 38		
4700	gm.	2nd basal diet
150	gm.	Liver Extract, Lilly
0.0015	gm.	biotin*
150	gm.	carcinogen solution (2% in cottonseed oil)

* Merck's crystalline biotin (synthetic).

As in the earlier experiment, the rats' livers were palpated at weekly intervals, and as soon as it was certain that a tumor was present the animal was killed and section taken for microscopic confirmation. Administration of the carcinogen ceased only with death of the animals.

RESULTS

The results obtained in this experiment are shown graphically in Fig. 1, and should be compared with Fig. 21 of our earlier paper. In both figures the ordinates represent cumulative tumor incidence in per cent, and

* Presented at the 37th Annual Meeting of the American Association for Cancer Research at Atlantic City, New Jersey, March 11, 1946.

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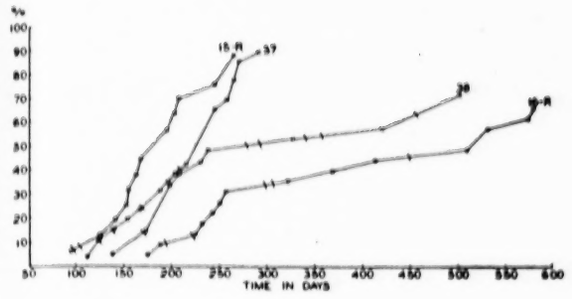

FIG. 1

the abscissae represent latent period in days. Death of tumor-free rats is indicated by a short line perpendicular to the graph of each diet, and the number adjacent to each curve is that of the diet concerned.

In Fig. 21 of our earlier paper (2) it is seen that 3 per cent of liver extract in the diet (No. 16) gives striking retardation of tumor development as compared with the control diet (No. 15), but 15 per cent of liver extract (No. 30) gives appreciably less protection.

In the present experiment, as shown by Fig. 1, the latent period of tumor development is somewhat prolonged and the slope of the curves is less steep, undoubtedly a result of reduction by one third of the concentration of the carcinogen in the diet. There is, however, a striking similarity between the curves for diets 38 and 30, and there seems little doubt that our explanation of the course of events noted with diet 30 is correct.

The results with diet 37 indicate that although biotin will accelerate liver tumor development on a diet that in itself protects against carcinogenesis, it will not have this effect when added to a diet that favors early carcinogenesis.

Not shown by the curve for diet 16R is the death of the last two rats in the experiment, both of which died tumor-free, one on the 615th day and the other on the 628th day.

Table II permits additional comparison of the original and subsequent experiments. The data in the first three lines have been taken from our earlier paper. It will be seen that the mortality rate during the period before the first tumor developed (ascertainable from columns 3 and 4) on diets 15R and 16R was no less than that on the original diets 15 and 16. Hence, the reduction of carcinogen content for the diets was fruitless, and had the undesirable effect of prolonging the latent period of tumor development.

TABLE II

Diet number	Date begun	Rats begun	Effective total	Rats developing tumors	50% Tumor incidence
					Days
15	May 19, 1941	55	34	30	139
16	"	40	28	28	401
30	Apr. 21, 1942	25	20	17	266
15R	May 18, 1944	45	16	14	183
16R	"	35	23	15	455
37	"	50	26	23	227
38	"	25	21	15	280

SUMMARY

Fifteen per cent of liver extract in the diet had given less protection against *p*-dimethylaminoazobenzene carcinogenesis than had 3 per cent, presumably because of the biotin content of the former diet. To test this point four diets were used: (1) control (favorable to early carcinogenesis); (2) control plus biotin; (3) control plus 3 per cent liver extract; and (4) control plus biotin plus 3 per cent liver extract. The biotin level in diets 2 and 4 approximated that of the 15 per cent liver diet. The curves of tumor development on diets 1, 3, and 4 were similar to those on the control, 3 per cent liver extract, and 15 per cent liver extract diets, respectively, of the earlier experiment. Diet 2 did not show accelerated tumor development. Thus, addition of biotin to a protective diet probably accelerated carcinogenesis, but addition of biotin to a diet favorable to early carcinogenesis did not have this effect.

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The Effect of Diet Containing Dried Egg Albumin upon *p*-Dimethylaminoazobenzene Carcinogenesis*

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(Received for publication October 12, 1946)

In 1941 Miller, Miner and Rusch (4) reported briefly that a diet containing 12 per cent of dried egg white retarded liver tumor production by *p*-dimethylaminoazobenzene, and remarked that egg white seemed to offer more protection than did casein. Since no additional information upon this subject had appeared, the experiment herein described was begun. Subsequently, Kline, Miller and Rusch (3) showed that the protection displayed by a diet containing 12 per cent of dried egg white was not due to avidin. Since their experiment was conducted differently from ours and was terminated at the end of six months, it seems worth presenting our data.

METHODS

As in our other experiments with *p*-dimethylaminoazobenzene (1, 2), the rats received the carcinogen-containing diet *ad libitum* until it was determined by palpation that a liver tumor was present. The animals were then killed and specimens taken for histologic examination.

The composition of the diets used is given in Table I.

RESULTS

The results are shown graphically in Fig. 1, in which diets 39 and 40 (each containing 15 per cent of dried egg albumin) are compared with low protein diet 15R (91 per cent rice) and diet 16R (diet 15 plus 3 per cent of Liver Extract, Lilly). These four groups of animals were contemporaneous and received the same concentration of carcinogen. As in our other papers, the cumulative tumor percentage is plotted against time in days, and death of a tumor-free animal is indicated by a short line perpendicular to the appropriate curve.

TABLE I. COMPOSITION OF DIETS

	Basal diets	
	No. 2	No. 6
Dried egg albumin*	0.0	150.0
Primex	50.0	50.0
McCollum's salt mixture No. 185 (Modified)	40.0	40.0
Carotene	0.01	0.01
Vitamin D Concentrate in Cottonseed Oil (400,000 U./gm.)	0.005	0.005
Thiamin	0.005	0.005
Riboflavin	0.001	0.0015
Vitamin B_6	0.003	0.003
Nicotinic acid	0.005	0.01
Distilled natural tocopherols	0.01	0.01
Calcium pantothenate	0.0056	0.0056
Choline chloride	0.0	1.0
Starch	0.0	759.0
Ground polished rice	910.0	0.0

* "Powdered Hen Egg Albumin," obtained from Henningsen Brothers, 99 Hudson Street, New York City.

Diet 15R
4850 gm. second basal diet
150 gm. carcinogen solution (2% in cottonseed oil)

Diet 16R
4700 gm. second basal diet
150 gm. Liver Extract, Lilly
150 gm. carcinogen solution (2% in cottonseed oil)

Diet 39
4850 gm. sixth basal diet
150 gm. carcinogen solution (2% in cottonseed oil)

Diet 40
4700 gm. sixth basal diet
150 gm. Liver Extract, Lilly
150 gm. carcinogen solution (2% in cottonseed oil)

* Presented at the 37th Annual Meeting of the American Association for Cancer Research at Atlantic City, New Jersey, March 11, 1946.

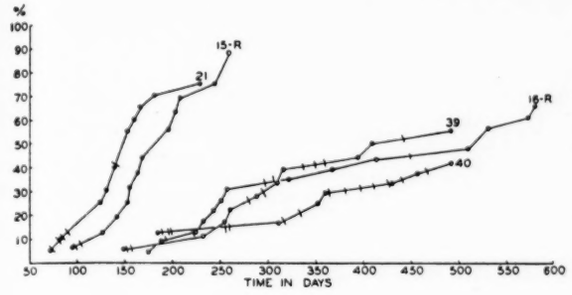

FIG. 1

We did not run an experiment with a diet differing from the albumin diet only in replacement of albumin by casein, but comparison of curves for diets 1 and 21 from our earlier data (1) with diet 39 gives a rough idea of the relative efficacy of casein and egg white in protecting against *p*-dimethylaminoazobenzene carcinogenesis. The curve for diet 21 is reproduced in Fig. 1 of this paper, and those for diets 1 and 21 are given in Figs. 18 and 24 of our earlier paper (1). Certain differences between these diets should be noted. Diets 1 and 21 contained 50 per cent more carcinogen than did 29, and consisted of 20 per cent casein. Diet 21 contained 1.5 μgm. of riboflavin per gm., and diet 1 contained 5 μgm. per gm. The albumin diet was patterned after diet 21, and was intended to contain 1.5 μgm. of riboflavin per gm., but an assay of the egg white revealed a riboflavin content of 21.6 μgm. per gm., making the riboflavin content of diet 39 actually 4.7 μgm. per gm., a level comparable with that of diet 1. In our original experiments (1), the curves for diets 15 and 21 nearly coincided. Presumably, if diet 21 had been repeated with a concentration of 0.6 mgm. of carcinogen per gram, the resultant curve should have approximated that of diet 15R.

Even after allowances are made for more rapid carcinogenesis on those diets containing 0.9 mgm. of carcinogen per gm., it is evident that the 15 per cent albumin diet afforded striking protection against carcinogenesis as compared with diets low in riboflavin and protein (diet 15R), low in riboflavin but containing 20 per cent casein (diet 21), and containing adequate riboflavin and 20 per cent caesin (diet 1). However, albumin offered no more protection than did the addition of 3 per cent of liver extract (diet 16R) to diet 15R. Incorporation of liver extract (diet 40) into diet 39 appeared to result in additional protection against the carcinogen. In view of the relatively large number of deaths of tumor-free animals on diet 40 as shown in Table II and Fig. 1, the significance of this difference is doubtful. However, there was a striking difference in the incidence of cirrhosis as revealed histologically in diets 39 and 40. Of 11 livers from diet 39 examined microscopically, 3 showed no cirrhosis, and of 12 livers from diet 40, ten showed no cirrhosis and two showed slight cirrhosis. There were also fewer deaths among rats on diet 40 during the latent period of carcinogenesis.

TABLE II

Diet No.	Date begun	Total rats used	Effective total	No. of rats with tumors	50% Tumor incidence, days
15R	May 18, 1944	45	16	14	183
16R	"	35	23	15	455
39	May 29, 1944	25	18	10	410
40	"	25	24	10	never
21	Sept. 11, 1941	30	20	15	153

DISCUSSION

No attempt was made to control the biotin or avidin content of the albumin diets since it was considered improbable that these two factors were significant. This opinion has been substantiated by Kline, Miller and Rusch (3). Furthermore, although biotin does have a procarcinogenic effect under proper conditions, our experience (2) leads us to believe that if a biotin-containing diet gave a tumor incidence comparable to that of diets 15R or 21, the removal of biotin would not alone suffice to cause the tumor incidence to approximate that of diet 39.

SUMMARY

Development of liver tumors in rats fed a diet containing 15 per cent of dried egg albumin and 0.06 per cent of *p*-dimethylaminoazobenzene was greatly retarded as compared with the development upon a diet favorable to tumor development. The degree of protection was greater than that provided by casein and riboflavin, and seemed slightly enhanced by addition of 3 per cent of liver extract.

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Etiologic Factors in Carcinoma of the Penis*

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(Received for publication November 12, 1946)

The main objective of this paper is to evaluate the significance of certain etiologic factors in carcinoma of the penis. The factors considered are venereal disease, circumcision, and the age and race of the patients.

A second objective is to illustrate the use of control groups in a statistical study. The use of controls is routine in experimental work and every experimental group is checked by one or more controls. In statistical studies on cancer, however, control groups are not as frequently used. This paper exemplifies several types of control groups and considers the necessity and advantages in the use of controls in statistical work.

Clinical and control groups.—The first table summarizes some statistics on the clinical and control groups studied. The clinical group under investigation consisted of 139 men with carcinoma of the penis. These patients were admitted to Hines Veterans Hospital during the 14 year period from 1931 to 1944. A detailed report on these men was published by Lenowitz and Graham (1).

The first control group, A, consisted of the 14,472 male patients admitted to the hospital with tumor during 1931 to 1944. For most purposes, this group is too large and cumbersome and has, therefore, limited value as a control.

A second control group, B, was composed of 236 men admitted during 1931 to 1944 with carcinoma of the kidney. This group was useful in studying the incidence of venereal disease. It could not be used, however, to determine the incidence of circumcision because these patients had not been questioned in regard to this factor.

A third group, C, was set up consisting of 209 patients who were admitted consecutively to the tumor clinic in 1944. These patients were interviewed by Mr. Philipp Zinkgraf for a study conducted by the U. S. Public Health Service.

The last group included 4 men with carcinoma of the penis and 2 Jewish men. For theoretical reasons, it seemed advisable to exclude these 6 patients leaving a new control group, D, of 203 men with tumor.

The number of colored men in the last control group was insufficient for some of the studies. Therefore, all colored men who happened to be in the hospital on a certain day in July, 1945 were interviewed. This procedure yielded 2 new control groups, E and F, consisting of 55 colored men with tumor and 113 with other diseases.

TABLE I: STATISTICAL DATA ON PATIENTS WITH CARCINOMA OF PENIS AND ON PATIENTS IN THE CONTROL GROUPS

Group	No. of patients	Year of admission	World War II veterans, %	Average age ± Standard deviation: All patients	Average age ± Standard deviation: Excluding World War II veterans
Clinical group					
Men with carcinoma of penis	139	1931-1944	0.0	49.26 ± 9.11	–
Control groups					
A. All men with tumor	14,472	1931-1944	3.8	–	–
B. Men with carcinoma of kidney	236	1931-1944	0.4	48.96 ± 6.64	–
C. Unselected men with tumor	209	1944	16.7*	50.96 ± 11.61	55.31* ± 6.74
D. Selected men with tumor	203	1944	16.7*	51.01 ± 11.58	55.36* ± 6.77
E. Colored men with tumor	55	1945	36.4*	46.23 ± 13.29	54.50* ± 6.67
F. Colored men with other diseases	113	1945	52.2*	40.60* ± 13.11	52.87* ± 5.34

* Percentage or average is significantly different from that for the clinical group (P is less than .01).

Age.—To understand the age distribution of the clinical and control groups, it is first necessary to consider the composition of the population from which the

* Presented at the Annual Meeting of the American Association of Pathologists and Bacteriologists March 8, 1946 (*Am. J. Path.*, 22:637-638. 1946).

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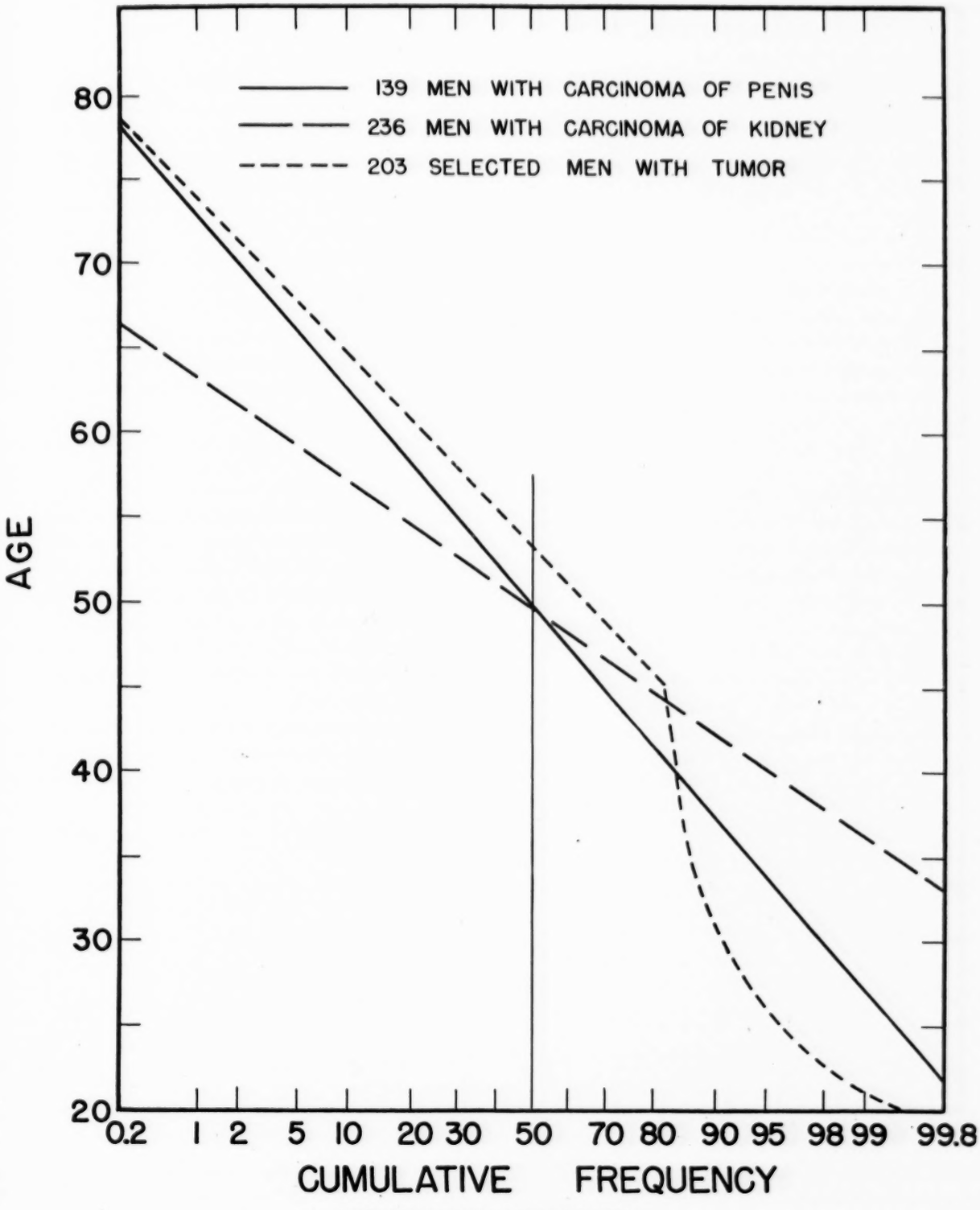
patients are derived. During 1931 to 1941, the patients of this hospital were obtained from a fixed group which consisted chiefly of veterans of World War I and to a lesser extent of Spanish American War veterans. The average age of the population from which the patients were derived was, therefore, increasing from



DESCRIPTION OF FIGURE 1

FIG. 1.—Cumulative frequency curve of age distribution of clinical group including 139 patients with carcinoma of penis. Curve plotted on arithmetic probability paper.

year to year. From 1942 to 1944 veterans of World War II were also admitted to the hospital and in rapidly increasing numbers.

The 139 patients with carcinoma of the penis were admitted during 1931 to 1944 and none of them were World War II veterans. The age distribution of the clinical group is represented in Fig. 1 by means of a cumulative frequency curve constructed on arithmetic probability paper. First of all, it is seen that the curve is a straight line. This finding indicates that the frequency curve is symmetrical or normal (2). The group had an average age of 49.26 years with a standard deviation of 9.11 years. The 39 colored men in the group had a significantly lower average age (46.35) and a lower standard deviation (5.25) than the white men (50.40 ± 10.00 years).

The control group, B, consisted of 236 men admitted during 1931 to 1944 with cancer of the kidney. Only one of the patients (0.4 per cent) served in World War II. The cumulative frequency curve (Fig. 1) is also a straight line. A comparison of this curve with that for the clinical group shows that the lines intersect at the point for 50 per cent and that the slope of the line for the control group is slightly less than that for the clinical group. The average age is 48.96 years which is practically identical with the 49.26 years for the clinical group. The standard deviation is 6.64 years which is significantly less than the 9.11 for the patients with cancer of the penis. The adequacy of this group B as a control is considered later.

Control group D consisted of 203 selected men with tumor who were admitted during 1944. The group had, therefore, a high percentage (16.7 per cent) of World War II veterans. The mixture of the young men from the second World War and the older veterans of the previous wars resulted in the irregular age distribution shown in Fig. 1. The curve for this group consists of two distinct parts. The first part representing 80 per cent of the patients in the group is a straight line. The second part of the curve is curvilinear and includes all of the World War II veterans. The curve exemplifies the value of this type of graph. It shows distinctly that the group of patients is derived from two distinct populations. In spite of the high percentage of World War II veterans the average age of the group (51.01 years) and the standard deviation (11.58) are not appreciably different from that for the clinical group (49.26 ± 9.11 years).

If the World War II veterans are excluded from this control group D, the age distribution has a cumulative frequency curve which is a straight line. The curve is distinctly higher but parallel to that for the clinical group. The average age of the subsidiary control group D′ is significantly higher (55.31 years) than that for the clinical group (49.26). It is to be noted that both groups were drawn from a fixed population which is gradually aging. The patients of the control group were admitted in 1944 while those for the clinical group during 1931 to 1944 (average date of admission was February, 1937). The men in the control group were admitted then, on the average, 7 years after those of the clinical group and were 6 years older.

In comparison with the clinical group, it should be noted that the control group D (selected men with tumor) includes a number of relatively young patients and that the subsidiary group F′ (group F but excluding World War II veterans) have a relatively high average age. The question arises now whether group D or D′ are adequate controls for the clinical group. In judging a control group, one has to take into consideration the factors that are being studied. In an investigation on the incidence of cancer, age has to be rigidly controlled. In this paper the incidence of cancer in the control groups was not studied. The factors that were considered included (a) the incidence of circumcision in infancy and boyhood, (b) the incidence of venereal disease and (c) the percentage of colored men. One would not expect that these factors should be greatly affected by minor variations in age. Therefore, it would seem that either group D or D′ would be adequate as a control. As a further precaution both groups D and D′ were used. In all cases, the statistical constants for group D and subgroup D′ were approximately the same in spite of the differences in the average ages. This finding supports the belief that age is not a vital factor in these studies.

Similar analyses were made on control group E, 55 colored men with tumor and F, 113 colored men without tumor. These groups have a high percentage of World War II veterans (36.4 and 52.2 per cent respectively) and low average ages (46.23 and 40.60 years). Excluding World War II veterans, the average ages (54.50 and 52.87 years) were approximately as high as that for the subsidiary control group D′ (55.31 years).

In determining the statistical constants for the factors studied in this paper, it was found that there were no large differences in the statistical constants for group E and F and the subgroups E′ and F′, in spite of the higher average ages for the subgroups. In one case, however, there did appear a minor, but interesting, difference between the groups for colored patients and the corresponding subsidiary groups which exclude World War II veterans (see Table IV).

It may be concluded from this analysis of the age distributions that the groups B, D, E, and F are adequate controls to the clinical groups for the particular factors considered in this paper.

Venereal disease.—Table II shows that a history of

syphilis was obtained in a greater percentage of patients in the clinical than in the control groups both for white men (32 and 7.6 per cent respectively) and for colored (67 and 17.5 per cent). Similarly gonorrhea was more frequent in men with cancer of the penis than in the control patients (36 and 22.0 per cent respectively for white men and 72 and 42.1 per cent for colored). In fact, the men with cancer of the penis had syphilis approximately 4 times as frequently, and gonorrhea about twice as frequently as that obtaining in the control groups. Only 48 per cent of the white patients with penile cancer and only 8 per cent of the colored were free of a history of venereal disease. These figures may be contrasted to 74.3 and 49.7 per cent of the white and colored men with negative histories for venereal disease in the control groups. Recalculation of Table II after excluding veterans of World War II did not appreciably affect the percentages in the table. The observed differences in the percentages do not appear to be the result of the selection of cases or to other fortuitous factors. The differences in the incidence of venereal disease in the clinical and control groups are statistically and biologically significant. Men with cancer of the penis have relatively a very high incidence of syphilis and a moderately high incidence of gonorrhea.

To aid in the evaluation of the finding of a correlation between cancer of the penis and venereal disease, it is necessary to consider the problem whether a patient with one venereal disease is more prone to develop the other. *A priori*, one would expect that there is a correlation in the incidence of syphilis and gonorrhea. The incidence of syphilis was calculated in patients with and without gonorrhea (Table III). In white patients of the control groups, syphilis occurred in a much higher

TABLE II: VENEREAL DISEASE IN PATIENTS WITH CARCINOMA OF PENIS AND IN CONTROL PATIENTS

Group	No. of patients	Percentage of patients with history of Syphilis	Gonorrhea	Both diseases	Neither disease
WHITE MEN					
Clinical group					
With carcinoma of penis	100	32	36	16	48
Control groups					
Total	409	7.6*	22.0*	3.9*	74.3
B. With carcinoma of kidney	221	9.0	26.2	4.1	69.7
D. Selected men with tumor	188	5.9	17.0	3.7	80.9
COLORED MEN					
Clinical group					
With carcinoma of penis	39	67	72	46	8
Control groups					
Total	183	17.5*†	42.1*†	9.3*†	49.7*†
B. With carcinoma of kidney	15	47	53	20	20
E. Negroes with tumor	55	18	44	11	49
F. Negroes with other diseases	113	13.3	39.8	7.1	54.0

* Percentage is significantly different from that for clinical group ($P < .01$).
† Percentage is significantly different from that for total of white control groups ($P < .01$).

TABLE III: INCIDENCE OF SYPHILIS IN MEN WITH HISTORY OF GONORRHEA

Group	No. of patients With gonorrhea	No. of patients Without gonorrhea	Percentage of men with syphilis With gonorrhea	Percentage of men with syphilis Without gonorrhea
WHITE MEN				
Clinical group				
Men with carcinoma of penis	36	64	44†	25
Control groups				
Total	90	319	17.8*	4.7
B. With carcinoma of kidney	58	163	16	6.7
D. Selected men with tumor	32	156	22	2.6
COLORED MEN				
Clinical group				
Men with carcinoma of penis	28	11	64	73
Control groups				
Total	76	106	21	14
B. Men with carcinoma of kidney	7	7	28	57
E. Colored men with tumor	24	31	25	13
F. Colored men with other diseases	45	68	18	10

* Percentage is significantly different from that for patients without gonorrhea ($P < .01$).
† Percentage is probably significantly different from that for patients without gonorrhea ($P = .01$ to $.05$).

percentage of men with gonorrhea than in those without (17.8 and 4.7 per cent respectively). The other percentages are based on small groups and are not definitely significant. The data support the *a priori* assumption that men with a history of gonorrhea have a relatively high incidence of syphilis.

Circumcision.—A detailed analysis of the incidence of circumcision is presented in Table IV. Of 39 colored and 100 white men with carcinoma of the penis, none were circumcised before the age of 6. In contrast, 17.9 per cent of the colored and 12.8 per cent of the white non-Jewish men in the control groups were circumcised early in life. The difference in the percentages for the clinical and control groups is statistically significant.

The incidence of circumcisions in white men during the ages of 6 to 16 years and 17 to 35 years was the same for the clinical (2 and 5 per cent) as for the control groups (2.1 and 3.7 per cent). The circumcisions during the ages 17 to 35 years occurred usually while the men were in the Army during World War I and II.

The findings suggest that circumcision performed early in life protected white and colored men against carcinoma of the penis but circumcision later in life had no effect on the incidence of penile cancer.

Is the low incidence of carcinoma of the penis in men circumcised early in life due to or associated with a low incidence of venereal disease? Table V shows that a negative history of venereal disease was given by 83 per cent of the white and 50 per cent of the colored men circumcised early in life, and by 82.9 and 52.8 per cent of the white and colored uncircumcised patients. Evidently men who were circumcised early in life had the same incidence of venereal disease as non-circumcised men. The non-occurrence of carcinoma of the penis

TABLE IV: CIRCUMCISION IN PATIENTS WITH CARCINOMA OF PENIS AND IN CONTROL PATIENTS

Group	No. of patients in group	Percentage of patients circumcised: At age of 0-5 years	At age of 6-16 years	At age of 17-35 years	Prior to admission 4-8 years	Prior to admission 0-3 years
WHITE MEN						
Clinical group						
Men with carcinoma of penis	100	0	2	5	3	21
Control group						
D. Selected men with tumor	188	12.8*	2.1	3.7	0.5	0.0*
COLORED MEN						
Clinical group						
Men with carcinoma of penis	39	0	0	3	3	18
Control groups						
Total	168	17.9*	3.0	3.6	0.0	3.0*
E. Colored men with tumor	55	24	2	0	0	4
F. Colored men with other diseases	113	15.0	3.5	5.3	0.0	2.7
WHITE MEN EXCLUDING WORLD WAR II VETERANS						
Control group D	154	11.7	2.6	4.5	0	0
COLORED MEN EXCLUDING WORLD WAR II VETERANS						
Control groups—total	89	24†	2	5	0	1

† Percentage is significantly higher than that for the corresponding white control group.
* Percentage is significantly different from that for the corresponding clinical group.

TABLE V: INCIDENCE OF VENEREAL DISEASE IN CIRCUMCISED AND NON-CIRCUMCISED MEN IN THE CONTROL GROUPS

Group	No. of patients in group	Percentage of patients with history of: Syphilis	Gonorrhea	Both diseases	Neither disease
WHITE MEN					
Control group					
D. Selected men with tumor					
Not circumcised	152	6.6	14.5	4.0	82.9
Circumcised at age of 0-5 years	24	0	17	0	83
Circumcised at age of 6-35 years	12	8	50	8	50
COLORED MEN					
Control groups					
Total, E and F					
Not circumcised	123	15.5	40.7	8.9	52.8
Circumcised at age of 0-5 years	30	13	43	7	50
Circumcised at age of 6-35 years	15	13	40	7	53

in circumcised men is not the result of a low incidence of venereal disease.

Race.—It is seen in Table VI that, of the 139 men with carcinoma of the penis, 28.1 per cent were colored. In contrast, the percentage of colored patients in the control groups was approximately 7 per cent or only one-fourth of that found for the clinical group. The difference in the percentage colored for the clinical and control groups is statistically significant. In another study (3) based on the mortality statistics of the United States for 1930 to 1934, the percentage colored for men with cancer of other genitourinary sites (chiefly penis) was significantly higher than that for all men with cancer (14.74 and 4.61 per cent, Table VI). The finding of a high percentage colored for carcinoma of the penis both in Hines Veterans Hospital and in the Mortality Statistics of the whole country indicates strongly that this high percentage is not only statistically but is biologically significant.

The relatively high percentage of colored patients in the group with carcinoma of the penis suggests that Negroes have a higher incidence of this tumor. The questions arise whether the frequency of this tumor in Negroes is due to (a) a lower incidence of circumcision in colored men, (b) a greater incidence of venereal disease, or (c) a racial susceptibility to penile cancer. These three factors can be tested statistically.

The relative incidence of circumcisions in white and colored men of the control groups is shown in Table IV. It is seen that, in the early age groups, the percentage of Negroes with circumcisions was slightly but not significantly higher than the corresponding percentages for the white men. Circumcisions early in life were found in 17.9 per cent of colored and 12.8 per cent of the white men. The difference in the percentages is not definitely significant. On excluding the World War II veterans, the colored men had a significantly higher incidence of early circumcisions than white men (24 and 11.7 per cent, Table IV). It may at first seem surprising that such a large number of colored men were circumcised during infancy or babyhood. The high incidence may be attributed to the tendency of interns and residents, particularly in the South, to circumcise colored infants born in hospitals. Incidentally, it may be pointed out that the figures show that this tendency is decreasing. The young colored veterans of World War II were circumcised less frequently as infants than the older colored veterans of World War I.

Lack of circumcision is then not the factor which is responsible for the high incidence of carcinoma of the penis among colored men.

TABLE VI: RACIAL DISTRIBUTION OF PATIENTS WITH CARCINOMA OF PENIS AND OF CONTROL PATIENTS

Group	No. of patients	Percentage colored
Clinical group		
With carcinoma of penis	139	28.1
Control groups		
A. All men with tumor	14,472	6.8*
B. With carcinoma of kidney	236	6.4*
D. Selected men with tumor	203	7.4*
Mortality Statistics, U. S. 1930-1934		
With cancer of other genitourinary sites	1,411	14.74
All men with cancer	278,860	4.61*

* Percentage is significantly different from that for corresponding clinical group.

The factor of venereal disease is considered in Table II. In the control groups, the colored men had approximately twice the incidence of syphilis as white men (17.5 and 7.6 per cent) and twice the incidence of gonorrhea (42.1 and 22.0 per cent). The difference in the percentages are significant. Only one half of the colored but three-fourths of the white men were free of venereal disease. Evidently Negroes contracted venereal disease about twice as frequently as white men.

Is the high incidence of venereal disease in colored patients responsible for the high incidence of carcinoma of the penis among Negroes? This problem was studied by analyzing the 14,472 men with tumor into

TABLE VII: ESTIMATED INCIDENCE OF CARCINOMA OF PENIS IN WHITE AND COLORED PATIENTS WITH AND WITHOUT VENEREAL DISEASE

Group	Estimated number of of men in group		Estimated percentage of men with carcinoma of penis	
	White	Colored	White	Colored
All men with tumor admitted 1931-1944	13,490	982	0.74	3.97
With tumor but without history of venereal disease	10,003	488	0.48	0.62
With tumor and history of syphilis	1,022	172	3.13	15.12
With tumor and history of gonorrhea	528	91	3.03	19.78
With tumor and history of both venereal diseases	2,968	413	1.21	6.78

groups with and without venereal disease and estimating the incidence of penile cancer in each group. The data presented in Table VII were not corrected for circumcised and Jewish patients, but these two factors did not affect the conclusions that may be drawn from the table.

Of the 14,472 patients from the tumor clinic, 10,003 white and 488 colored men were, it is estimated, free of venereal disease. Of these 0.48 per cent of the white and 0.62 per cent of the colored patients are believed to have had cancer of the penis. The difference in the percentages is not statistically significant. From this finding it would seem that white and colored men who do not develop venereal disease are equally susceptible to carcinoma of the penis. There is then no evidence of any unusual susceptibility of Negroes to carcinoma of the penis or of any immunity of white men to this lesion.

In 1,022 white men with a history of syphilis approximately 3 per cent had cancer of the penis, while in 172 colored syphilitic patients 15 per cent had penile cancer. Similarly, 1.21 per cent of the white and 6.78 per cent of the colored men with gonorrhea had, it is calculated, carcinoma of penis. Even if the factors of syphilis and gonorrhea are controlled, the colored men still had a higher incidence of this tumor. Evidently venereal disease by itself did not account for the high incidence of carcinoma of the penis in colored men.

DISCUSSION

Venereal disease.—It has been shown that patients with carcinoma of the penis have a very high incidence of venereal disease. Evidently there is a correlation between the tumor and the infection. This finding can be interpreted in one of two ways. Either syphilis and gonorrhea are predisposing causes of carcinoma of the penis or there is some other factor that predisposes both to venereal disease and to penile cancer. Statistics by itself cannot give an answer as to which interpretation is correct. From a knowledge of the pathogenesis of these diseases, it seems evident that gonorrhea is not a direct cause of cancer of the penis. Presumably some other factor predisposes both to gonorrhea and to cancer of the penis.

Racial factors.—Carcinoma of the penis occurs relatively frequently in Negroes, is infrequent in white, non-Jewish men and is practically unknown in Jews. Is this distribution due to inherited racial charactistics or is it dependent on environmental factors?

Carcinoma of the penis has not been observed in non-Jewish men who were circumcised early in life. Presumably, the absence of cancer of the penis in Jews is explained by their religious practice of circumcision eight days after birth. It does not then seem necessary to formulate the hypothesis of an inherited racial immunity.

The high incidence of carcinoma of the penis in Negroes raises the question of racial susceptibility. The present data have shown that colored men free of venereal disease have penile cancer as infrequently as white men. There did not seem to be a racial susceptibility in colored men free of venereal disease. The findings suggest that environmental, not racial, factors determine the incidence of carcinoma of the penis.

Poor sex hygiene.—Since venereal disease and racial immunity have been excluded as being primary etiologic factors in cancer of the penis, the question arises whether poor sex hygiene is a predisposing cause. Poor sex hygiene is not, of course, a single factor but consists of a group of correlated habits such as promiscuity, lack of prophylaxis, infrequent or inadequent washing, and failure to obtain early medical treatment for minor inflammatory penile lesions.

It seems proper to judge the sex hygiene of a group of men by the incidence of venereal disease. Its high incidence among colored men is an indication of the poor sex habits practiced by many Negroes. The high incidence of both syphilis and gonorrhea in men with cancer of the penis is probably an indication that patients with penile cancer had practiced poor habits. Apparently, poor sex hygiene predisposes both to venereal disease and to carcinoma of the penis.

In venereal disease, the primary disposing factors are promiscuity and lack of phophylaxis. In cancer of the penis, the primary predisposing factors may be such factors in sex hygiene as the infrequent or inadequate washing of the genitalia and the improper care of inflammatory penile lesions.

The hypothesis that poor sex hygiene is a predisposing factor in penile cancer explains the high incidence of venereal disease and the high percentage of Negroes in the 139 patients studied.

Circumcision.—One of the most interesting findings of the present study is that circumcision early in life apparently protects against carcinoma of the penis but circumcision during boyhood or manhood has no appreciable effect on the incidence of the tumor. It would have been of interest to know the exact reasons for the circumcisions in the clinical and the control groups, but this information was not available.

What protects men circumcised early in life against penile cancer? That they do not practice any better sex hygiene is indicated by the incidence of venereal disease among these men. It is possible that circumcision lessens the accumulation of smegma and dirt and in this way inhibits cancer. If this hypothesis is correct, one would expect that circumcision during boyhood should also protect against cancer of the penis. There is a possibility that the individual circumcised early in life is protected against penile cancer not by

cleanliness but by some other process, the nature of which is not known.

There is, then, a factor—circumcision—which occurs in the infant and which affects the incidence of tumor in the adult. Perhaps other factors operating in infancy determine whether the adult develops other types of cancer. When the clinician or the research worker takes a history of a patient with cancer of the stomach, for example, the patient is questioned with regard to occupation, diet, and other factors that affect the adult. Perhaps it would be more important to ask about the patient's feeding habits as an infant.

Etiologic factors.—The present findings suggest that there are two fundamental etiologic factors in carcinoma of the penis. The first is circumcision in early life. This seems to offer by an unknown method almost complete protection against penile cancer later in life. This factor suffices to explain the almost complete absence of this tumor in Jewish men. The second etiologic factor, poor sex hygiene, seems to be responsible for the following statistical findings: (a) the high incidence of venereal disease in men with carcinoma of the penis, (b) the relatively greater frequency of carcinoma of the penis in colored men, (c) the high incidence of venereal disease in colored men, and (d) the high incidence of syphilis in men with a history of gonorrhea. It may be concluded that carcinoma of the penis can be prevented either by good sex hygiene or by circumcision early in life.

Control groups.—A previous paper (4) has presented a discussion on the types of control groups that may be used in statistical studies in cancer. This work exemplifies the use of several different types of control groups. A review of Tables I to IV shows that there was good agreement in the data obtained for the different control groups.

It should be noted in passing that the data on venereal disease and circumcision in patients with carcinoma of the penis (Tables II and IV) are, by themselves, interesting and descriptive. No conclusions, however, can be drawn when one studies the clinical group by itself. A comparison of the clinical and the control groups, as in Tables II and IV, leads to conclusions that are almost as clear-cut and decisive as in a planned experiment.

SUMMARY AND CONCLUSION

As compared with the control groups, the group with carcinoma of the penis contained a high percentage of Negroes, had a high incidence of syphilis and gonorrhea, a very low incidence of circumcisions early in life and the same incidence of circumcision during boyhood and early manhood. Positive correlations were obtained for the factors carcinoma of the penis, syphilis, gonorrhea, and the colored race. The common denominator in these four factors appears to be poor sex hygiene. The incidence of carcinoma of the penis could be reduced either by early circumcision or by good hygiene.

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2. Schrek, R. Logarithmic Frequency Distributions. Human Biol., **13**:1-22. 1941.
3. Schrek, R. The Racial Distribution of Cancer. II. Tumors of the Kidney, Bladder, and Male Genital Organs. Ann. Surg., **120**:809-812. 1944.
4. Schrek, R., and Allaben, G. R. Statistical Analysis of 2,407 Admissions to the Tumor Clinic of Veterans Hospital, Hines, Illinois. Cancer Research, **5**:539-546. 1945.

Abstracts

Reports of Research

On the Production of Sarcoma with Wheat Germ Oil. HARRIS, P. N. [Eli Lilly and Co., Indianapolis, Ind.] *Cancer Research,* **7**:26-34. 1947.

Although treatment was prolonged and large amounts of oil were given, no tumors were obtained as a result of oral administration of ether-extracted wheat germ oil to rats. Following repeated subcutaneous or intraperitoneal injections of various types of wheat germ oil, sarcomas were obtained in 2 mice and 16 rats. The tumor percentage for mice was 5% of the effective total, and for rats was 8% of the effective total injected.—Author's abstract.

Production of Sarcoma in Rats with Light Green SF. HARRIS, P. N. [Eli Lilly & Co., Indianapolis, Ind.] *Cancer Research,* **7**:35-36. 1947.

Subcutaneous injection into the same region of an aqueous solution of the dye light green SF twice weekly for a period of 33 weeks resulted in development of sarcomas at the site of injection in 15 of an effective total of 24 rats. During the first month a 2% solution was used, but thereafter, the concentration was increased to 3%. The latent period varied from 35 to 85 weeks.—Author's abstract.

Reciprocal Effects of Natural Immune Bodies from Chickens and Ducks on Variants of a Sarcoma Virus. DURAN-REYNALS, F., and KING, J. W. [Yale Univ. Sch. of Med., New Haven, Conn.] *Cancer Research,* **7**:21-25. 1947.

This investigation was carried out to determine, first, the nature of the resistance of chickens and ducks to the Rous chicken sarcoma virus, and its variants in ducks, and second, the nature of the changes that took place in the virus in the process of mutation or variation. The immune bodies that develop naturally in the blood of aging chickens neutralize both the viruses of the chicken tumor and its duck variants as tested on both chickens and ducks. Therefore, the resistance of chickens against these viruses is linked with the presence of humoral factors and might be explained on this basis. On the contrary, comparable immune bodies from aging ducks neutralize only duck tumor viruses as tested on ducks, but not as tested on chickens; nor do they neutralize chicken tumor viruses as tested on either chickens or ducks. Therefore, the refractory state of ducks against heterologous chicken viruses, solidly established very shortly after hatching, cannot be linked with humoral factors but rather belongs to the so-called species resistance. However, the resistance of these hosts against the virus after it has varied and has become a duck virus is linked with humoral factors and might be explained on this basis. One may add that, with the tumor viruses here studied, the species resistance is far more effective than is the humoral resistance, and that the presence of natural specific immune bodies against the viruses is an indication of actual or potential susceptibility to these viruses. Analysis of the 8 possible sequences that can be obtained by testing the effect of chicken and duck sera against homologous and heterologous tumor viruses on homologous and heterologous hosts indicates that a pronounced antigenic change has taken place in the virus in the process of variation.—Authors' abstract.

Growth of Avian Tumors Other Than the Rous Sarcoma in the Anterior Chamber of the Guinea Pig Eye. SHRIGLEY, E. W., GREENE, H.S.N., and DURAN-REYNALS, F., [Yale Univ. Sch. of Med., New Haven, Conn.] *Cancer Research,* **7**:15-20. 1947.

It has been found that the Rous sarcoma is not the only avian tumor that will grow in the anterior chamber of the guinea pig eye. Two spontaneous growths originating in chickens and 3 methylcholanthrene-induced sarcomas, from chicken, guinea fowl and pigeon respectively, have been found to be transplantable to the eye of the guinea pig. Like the Rous sarcoma, these tumors do not grow to such a size as to completely fill the chamber. In general, tissue fragments in the alien host grow for 10 to 20 days and then regress in size over varying periods of time. However, in the case of one slow-growing spontaneous chicken tumor healthy tissue was recovered from the rodent's eye 83 days after inoculation.—Authors' abstract.

The Role of Heredity in the Etiology of Cancer. MARTYNOVA, R. P. *Bull. Exper. Biol. & Med.,* **19**:12-15. 1945.

The author mentions the role of heredity in certain tumors of fish, insects and mammals, but suggests that definite relationship between heredity and cancer has not been established conclusively. She assumes that if cancer is a hereditary disease, susceptibility to cancer in identical (monozygotic) twins is equal and this susceptibility should differ in fraternal (dizygotic) twins. If there is no difference in morbidity between the two groups, she feels that it proves that the hereditary factory is insignificant in cancer etiology. The author bases her report on 478 pairs of twins with cancer included among 45,000 tumor patients collected from 20 cancer institutes in the USSR. Reliable data was obtained only on 126 sets of twins, of whom 31 were identical and 95 fraternal. Among the former, cancer was found in 4 pairs of twins, or 12.9%, and among the latter in 13 pairs or 13.7%. The difference is not significant and a hereditary disposition, based on these figures is not established. She concludes that cancer cannot develop without mutation of a germ or somatic cell, and is not hereditary since somatic mutation is the precursor in the great majority of cases. A detailed theoretical discussion on mutation is included. —J. H.

Cholesterol Content of the Urine in Patients with Cancer. BRUGER, M. [New York Post Graduate Med. Sch. and Hosp., Columbia Univ., New York, N. Y.] *Arch. Int. Med.,* **72**:108-114. 1943.

The excretion of cholesterol in the urine in 26 normal subjects ranged from 0.27 to 3.88 mgm. in 24 hours with a mean of 1.69 ± 0.85 mgm. In 28 patients with a variety

of clinical disorders other than cancer, the mean excretion of cholesterol was slightly higher than in the normal subjects (2.01 mgm. in 24 hours), but for the most part the individual variations fell well within the normal range. In 8 of 32 patients with cancer, the excretion of cholesterol in the urine was significantly elevated above normal; the highest value noted was 47.8 mgm. in 24 hours in a patient with adenocarcinoma of the rectum. The mean amount of urinary cholesterol for this group was 6.09 mgm. in 24 hours. No correlation was observed between the sedimentation rate, the plasma cholesterol content and the degree of cholesteroluria. Hypercholesteroluria occurs independently of loss of protein in the urine; some theoretic considerations are offered to account for this phenomenon.—Author's summary. (J. G. K.).

A Study of the Effect of Certain Dietary Factors on the Production of Tar-Carcinoma in Mice. CAMERON, A. T., MELTZER, S., and LEDERMAN, J. M. [Univ. of Manitoba, Canada] *Canad. J. Research,* **23, Sec. E**:50-69. 1945.

The development of carcinoma in mice painted with gasworks tar or with 3,4-benzpyrene was not affected by varying the protein content of the Waddell-Steenbock diet from approximately 14% to 26%. When 1,2,5,6-dibenzanthracene was used as the tarring agent, the results were inconclusive because of the delay in onset of carcinoma. Inconclusive results were also obtained in an experiment designed to study the effect of the dietary content of essential unsaturated fatty acids on 1,2,5,6-dibenzanthracene carcinoma.—M. H. P.

The Solubilization of Polycyclic Aromatic Hydrocarbons by Purines. WEIL-MALHERBE, H. [North of England Council of the British Empire Cancer Campaign, Royal Victoria Infirmary, Newcastle upon Tyne, England] *Biochem. J.*, **40**:351-363. 1946.

Aqueous solutions of caffeine were shaken with solid 3,4-benzpyrene, filtered, passed through alumina and the benzpyrene estimated by fluorimetry. Experiments were also carried out on other polycyclic aromatic hydrocarbons, as well as about 40 purines and purine derivatives. The effects of varying the concentration of purine over a very wide range were also studied.

The discussion is on the mathematical relation of solubility change to concentration, the theory of the mechanism of solubility, constitution and solvent activity, and a comparison of choleic-acid with other hydrocarbon solvents.—I. H.

Effects of Purines on Fluorescent Solutions. WEIL-MALHERBE, H. [North of England Council of the British Empire Cancer Campaign, Royal Victoria Infirmary, Newcastle upon Tyne, England] *Biochem. J.*, **40**: 363-368. 1946.

The fluorescence of 3,4-benzpyrene (1μgm./ml.) in 1% aqueous caffeine solution is reduced to 87% by addition of 0.001 normal H_2SO_4, to 13.5% by 0.02 normal H_2SO_4 and not influenced by 0.2 normal H_2SO_4. This effect is reversible on neutralization of the acid. The compounds resulting in this reduction have to be present in great excess (many thousands of molecules per molecule of fluorescent compound). These observations have been extended by studying the inhibitory effect of different (1) purines and purine derivatives, (2) acids, (3) solvents, and (4) polycyclic aromatic hydrocarbons. The effects of these agents on fluorescent compounds other than hydrocarbons has also been studied. The physicochemical theory and relation of constitution to inhibitory activity are discussed.—I. H.

Succinic Dehydrogenase and Cytochrome Oxidase in Epidermal Carcinogenesis Induced by Methylcholanthrene in Mice. CARRUTHERS, C., and SUNTZEFF, V. [Barnard Free Skin and Cancer Hosp., and Washington Univ. Sch. of Med., St. Louis, Mo.] *Cancer Research,* **7**:9-14. 1947.

The activity of succinic dehydrogenase and cytochrome oxidase in the epidermis of mice undergoing carcinogenesis by methylcholanthrene has been investigated. The activity of succinic dehydrogenase in normal and in hyperplastic (methylcholanthrene-treated) epidermis is the same, but almost a four-fold increase occurs in the activity of this enzyme when the cells become carcinomatous. On the other hand the cytochrome oxidase activity of hyperplastic epidermis (after 6 and 12 paintings on alternate days with methylcholanthrene) was slightly greater than normal, but the activity of late hyperplastic epidermis (18 and 24 applications of the carcinogen) was nearly twice that of normal. In the carcinoma the activity of this enzyme was less than that of late hyperplastic epidermis, but greater than that of normal. Benzene, the solvent for the carcinogen, had a slight inhibitory effect upon the activity of both enzymes. The relationship of the enzymes with the metals, calcium, iron, copper, and zinc, is briefly discussed. Two tables and one figure are appended.—Authors' abstract.

The Inhibition of Urease and Succinoxidase by Metabolic Products of *p*-Dimethylaminoazobenzene and by Some Related Amines. ELSON, L. A., and HOCH-LIGETI, C. [Royal Cancer Hosp., London, England] *Biochem. J.*, **40**:380. 1946.

The compounds tested were aniline, *p*-aminophenol, *o*-, *m*-, and *p*-phenylenediamine, *as*-dimethyl-*p*-phenylenediamine, *as*-diethyl-*p*-phenylenediamine, *N*:*N*:*N'*:*N'*-tetramethyl-*p*-phenylenediamine, benzidine, 2:4'-diamino-5-dimethylaminodiphenyl, and Bindschedler's green. Rat organs were used and included the liver, kidney, spleen, testis, also tumors of the liver. The inhibition of urease by those compounds which are oxidized on exposure to air increased to a maximum during such exposure, indicating that the actual inhibitor is an intermediate product. Aniline and benzidine, which are not readily oxidized thus, are not inhibitory. All the *p*-diamines tested, and *o*-phenylenediamine, are strongly inhibitory; *m*-phenylenediamine is only slightly active. In general, those amines which inhibit urease also inhibit succinoxidase, but with the latter agent the oxidation of the amines by the cytochrome system is so rapid that maximum inhibition is obtained at once and a decrease in activity takes place on exposure to air. In the succinoxidase system with addition of cytochrome *c* the higher concentrations of the inhibitory amines (10^{-3}-10^{-4}M) generally give initially an increase in O_2 uptake followed by complete inhibition of the enzyme activity. With lower concentrations (10^{-5}-10^{-6}M) only a gradually increasing inhibitory effect is observed. Without addition of cytochrome *c* the results are less uniform in that the higher concentrations cause inhibition only in some of the livers; the lower concentrations are often inactive. The succinoxidase of the liver of animals which had been fed *p*-dimethylaminoazobenzene, even in tumor-free parts of the liver of tumor-bearing animals, showed the same general behavior towards the amines as that of control animals. On tissues of low oxidative capacity (spleen,

testis, Walker sarcoma, liver tumors induced by *p*-dimethylaminoazobenzene and 2-acetamidofluorene) no inhibitory effect on the O_2 uptake in presence of succinate is produced by amines that inhibit tissues of high succinoxidase content.—E. L. K.

A Note on the Action of Tannin upon Tumour Glycolysis. LASNITZKI, A. [Cancer Research Lab., Med. Sch., Univ. of Birmingham, Birmingham, England] *Biochem. J.*, **40**:263-264. 1946.

Tannic acid (0.1%) added to slices of the Jensen rat sarcoma, inhibited anerobic glycolysis (CO_2 production in an atmosphere of N_2 plus 5% CO_2) by 40%. It is suggested that (1) the tannic acid acts by dehydrating the enzyme proteins, (2) the activity of the enzymes is dependent upon the degree of hydration, and (3) the high water content of rapidly proliferating tissues facilitates the activity of the glycolytic enzymes.—I. H.

Discussion on Leukaemia and Leukosis in Man and Animals. ENGELBRETH-HOLM, J. [London, England] *Proc. Roy. Soc. Med.*, **39**:735-740. 1946.

The discussion opened with a paper on the comparative pathology of animal leukosis by Engelbreth-Holm, who regards the term "leukosis" as properly confined to true autonomous and malignant growths arising from the hemopoietic tissues. Speaking on avian leukemia, Blakemore discussed the connection between this condition and both visceral and neural lymphomatosis, in relation to the unitary theory of Biester and DeVries (*Diseases of Poultry*, Iowa, 1944) which attributes these diseases to a single virus and classifies them as the "leukosis complex." In Blakemore's experience, the neurolymphomatosis virus is much more infective for chicks than for older stock. It can be increased in virulence by rapid animal passage and then the agent produces a markedly different disease characterized by necrosis of the capillary walls.

Other contributors included E. G. White, who discussed leukemia in dogs; J. M. Alston, R. J. Ludford, G. R. Cameron and L. Foulds also contributed to the discussion. —R. H.

Desoxyribose Nucleic Acid from Isolated Chromosome Threads in Experimental Epidermal Methylcholanthrene Carcinogenesis in Mice. GOPAL-AYENGAR, A. R., and COWDRY, E. V. [Barnard Free Skin & Cancer Hosp., and Washington Univ. Sch. of Med., St. Louis, Mo.] *Cancer Research*, **7**:1-8. 1947.

Disruption of nuclear membranes releases into the surrounding medium the chromosomal elements which can then be isolated, concentrated and purified by differential centrifugation. Chromosomes of Swiss mice were thus isolated from normal epidermis, epidermis rendered hyperplastic by methylcholanthrene, and chromosomes from a transplantable squamous cell carcinoma. The concentrations of desoxyribose nucleic acid in the masses of isolated chromosomes were determined. In hyperplastic epidermis the concentration was less than in normal epidermis, whereas in squamous cell carcinoma it was greater.—Authors' abstract.

Relative Sensitivity of Chromosomes to Neutrons and X-Rays. III. Comparison of Carcinoma and Lymphosarcoma in the Rat. MARSHAK, A., and BRADLEY, M., *Proc. Nat. Acad. Sc.*, **31**:84-90. 1945.

Survival curves (log % normal vs. dose) for chromosomes of *Vicia faba* root tips, lymphoma (mouse), lymphosarcoma and carcinoma (rat) obtained at the intervals 3, 8, 12, 18, and 24 hours after treatment with either x-rays or neutrons all fit the equation $Y = e^{-kx}$. When the slopes (k) are plotted as a function of time there is a progressive decrease in k with time in all tissues studied except at the 12-hour interval where there is either a plateau as in the cases *V. faba*, lymphosarcoma, carcinoma, or a peak as found with the lymphoma. It is inferred from this observation that the stage in the nuclear cycle occurring at the time of the 12-hour interval represents a critical period in the physiology of all chromosomes studied.

Relative efficiency of neutrons and x-rays in producing chromosome damage is evaluated from the ratio of k obtained with x-rays (n/x) at each time interval. In all animal tissues and all plant species studied $n/x = 6$ for the phase of the nuclear cycle, which reaches anaphase 3 hours after irradiation. In contrast to this marked uniformity there is striking variation n/x in other phases of the nuclear resting stage not only between species but in a single type of tissue in any one species. Changes in n/x with time in any one type of cell may be used to identify phases in physiological activity of chromosomes in different parts of the resting stage. In terms of the ratio n/x, chromosomes of lymphosarcoma of the rat behave more like those of lymphoma in mouse than chromosomes of carcinoma of the rat. These results are taken to indicate that in the process of differentiation during ontogeny and also during "dedifferentiation" in carcinogenesis the chromosomes become altered in their physiology. One cannot therefore infer that the genetic theory which requires that the genome remain constant during embryogeny also implies that the chromosomes remain unchanged.—Authors' abstract.

Plant Tumours Induced by the Combined Action of Wounds and Virus. BLACK, L. M. [Rockefeller Inst. for Med. Research, Princeton, N. J.] *Nature, London*, **158**:56-57. 1946.

Plant tumors can be induced in numerous host species by a virus previously described (L. M. Black, *Am. J. Bot.*, **32**:408. 1945), and experiments have now confirmed the earlier suggestion that such tumors arise in association with the wounding of plant tissues. That the tumor tissue is capable of indefinite growth without the differentiation of normal plant organs was shown by grafting fragments of the growth to healthy plants, and by growth *in vitro* on White's medium without production of roots, stems or leaves. Two figures illustrate the gross and microscopic appearances of such tumors on the roots of infected sweet clover.—A. H.

Clinical and Pathological Reports

Clinical investigations are sometimes included under Reports of Research

Multiple Tumors

The Multiplicity of Origin of Malignant Tumors. Collective Review. Slaughter, D. P. [Univ. of Ill., Coll. of Med., Research and Educational, and Presbyterian Hosps., Chicago, Ill.] *Surg., Gynec. & Obst., Internat. Abst.*, **79**:89-98. 1944.

The problem of multiple tumors is discussed from three standpoints to determine what, if any, are their patterns of localization: (1) the microscopic origin of single tumors, (2) the benign and malignant tumors known or generally considered to be of multiple origin, and (3) an analysis of tabulated cases of double primary carcinomas.—J. G. K.

Diagnosis

The Vaginal Smear in the Diagnosis of Uterine Cancer. Fremont-Smith, M., Graham, R. M., Janzen, L. T., and Meigs, J. V. [Vincent Memorial Hosp. of Massachusetts General Hosp., and Harvard Med. Sch. Boston, Mass.] *J. Clin. Endocrinol.*, **5**:40-41. 1945.

This is a brief report calling attention to the usefulness of vaginal smears in the diagnosis of uterine cancer and is based upon a study of this material from 813 women. The authors conclude that cancer even in the early stages can be diagnosed by this method. A positive smear is presumptive evidence of cancer and the patient should have a biopsy immediately despite any lack of clinical evidence of disease. A negative smear does not exclude the possibility of cancer but is strong evidence against its presence.—J. B. H.

Therapy—General

Some Effects of Testosterone, Testosterone Propionate, Methyl Testosterone, Stilbestrol, and X-Ray Therapy in a Patient with Cushing's Syndrome. Deakins, M. L., Friedgood, H. B., and Ferrebee, J. W. [Harvard Med. Sch., and Peter Bent Brigham Hosp., Boston, Mass.] *J. Clin. Endocrinol.*, **4**:376-384. 1944.

In a 15 year-old girl with clinical and laboratory evidence of Cushing's disease, androgenic treatment in the form of daily intramuscular injections of 25 mgm. of testosterone propionate or of daily oral doses of 40 mgm. of methyl testosterone for 16 days, did not produce demonstrable clinical benefits but did increase the acne, hirsutism, and general virilism of the patient. Notwithstanding the lack of clinical benefit, the treatment with testosterone propionate brought about a positive nitrogen balance amounting to several grams daily and diminished considerably the excretion of creatine. Treatment with methyl testosterone in contrast to that with testosterone propionate produced a still greater retention of nitrogen and an increase rather than a decrease in creatinuria. Daily intramuscular injections of large amounts (2 to 10 mgm.) of diethylstilbestrol were of no benefit clinically and were accompanied by nausea and a transient hyperglycemia. Irradiation of the pituitary gland was followed by lessening of the virilism and of the plethora and by the appearance of normal menstrual periods.—J. B. H.

A Case of Cushing's Syndrome Treated with Testosterone Propionate. Whitelaw, M. J. [Charles S. Wilson Memorial Hosp., Johnson City, N. Y.] *J. Clin. Endocrinol.*, **4**:480-482. 1944.

A 17 year-old boy with indubitable evidence of Cushing's disease received treatment with intramuscular injections of 25 mgm. of testosterone propionate daily or on alternate days. This was followed by a noticeable gain in strength and increase in creatinuria plus slight augmentation of the excretion of creatinine, an elevation of blood levels of creatine, and an increase in libido. The osteoporosis, hypertension and excretion of calcium remained unchanged.—J. B. H.

Treatment of Breast Cancer with Testosterone Propionate. A Preliminary Report. Fels, E. [Buenos Aires, Argentina] *J. Clin. Endocrinol.*, **4**:121-125. 1944.

Varying degrees of benefit in 3 women with mammary cancer are stated to have followed treatment with intramuscular injections every other day of 25 mgm. of testosterone propionate. Case 1, a 34 year-old woman, had an alveolar type of mammary carcinoma along with a large uterine fibroma. Roentgenographic examination in October, 1941, showed evidence of infiltration of the hilum of the right lung but no skeletal metastases. Upon radical mastectomy in November, 1941, the presence of epitheliomas in the right axillary lymph glands was revealed. Roentgen-ray treatment was then given. In July, 1942, widespread metastases to the skeleton were demonstrable by x-ray. By July, 1943, when the patient was cachectic, unable to move her head, and suffering great pain despite daily dosage with 0.15 to 0.2 gram of morphine, treatment with testosterone propionate was begun. After 250 mgm. of testosterone propionate had been received, vomiting and pain became less. After receipt of 700 mgm. the fibroma had disappeared and metastatic nodules in the supraclavicular and cervical regions (apparently derived from the mammary cancer) had decreased in volume. The patient became ambulatory, regained her appetite, and showed signs of virilism. Roentgenographic examination in September, 1943, indicated no change in the status of skeletal metastases, but by December, 1943, there was considerable improvement and general calcification about the sites of metastases in the skeleton. In December, 1943, a cervical node was excised and compared histologically with an axillary node removed 2 years before. Now there was a much higher proportion of fibrous tissue and some walling off of the nests of cancerous tissue, an effect to which the author attributes the beneficial action of the endocrine treatment.

Cases 2 and 3 are reported in less detail and although they were stated to have been benefited by androgenic treatment, improvement does not seem to have been as noticeable as in Case 1.—J. B. H.

Treatment of Carcinoma of the Cervix by Interstitial Radium Needles at the Rhode Island Hosp. Supplemental Report. Waterman, G. W., and DiLeone, R. [Providence, R. I.] *Am. J. Obst. & Gynec.*, **50**:482-488. 1945.

Survival rates for the 5 and 10 year intervals of 309 cases previously reported are given. Of the whole group

100 patients survived for at least 5 years, and 69 of these survived for 10. Recently 5 year survival rates have been better due to the use of deep x-ray as a mode of treatment. Methods of therapy are given.—A. K.

Sclerosing Agent in Treatment of Subluxation of Mandible and of Hemangiomas of Mouth. SALMAN, I. [U. S. N. R.] *U. S. Nav. M. Bull.,* **44**:361-369. 1946.

Report of favorable results obtained by injecting hemangiomas of the oral cavity with a sclerosing solution.—C. W.

SKIN AND SUBCUTANEOUS TISSUES

Le traitement des cancers primitifs de la peau et des orifices cutanéo-muqueux à la Foundation Curie. [The Treatment at the Curie Foundation of Primary Cancers of the Skin and Muco-Cutaneous Orifices.] TAILHEFER, A., and COURTIAL, J. [Univ. of Paris, Paris, France] *Bull. Assoc. franç. p. l'étude du cancer,* **31**:85-115. 1943.

Skin epitheliomas and epitheliomas of the mucocutaneous orifices both have a variable prognosis, depending on their localization and their extension. There is no sharp difference between basal-cell and spinus-cell epitheliomas as far as their radio-sensitivity is concerned. The different kinds of treatment are discussed. When metastases are present, the authors prefer surgery and complete removal of the invaded lymph nodes. Where there is a recurrence of the cancer *in situ*, radiotherapy is indicated if surgery was applied first; surgery is indicated if radiotherapy was the initial treatment.—R. J.

Basal-Cell Carcinoma of Face, with Remote Metastases and Pathological Fractures. SINGER, A. [Oldchurch County Hosp., Romford, England.] *Brit. J. Surg.,* **32**:537-538. 1945.

The primary tumor, which had caused extensive ulceration of the face, had an unequivocal appearance of basal-cell carcinoma. Metastases of the same histological character were present in both femors, which were fractured, and in the ilium.—E. L. K.

Multiple Idiopathic Hemorrhagic Sarcoma of Kaposi in a Full-Blooded Negro. PERSKY, B. P., and LISA, J. R. [City Hosp., Welfare Island, Dept. of Hosps., New York, N. Y.] *Arch. Dermat. & Syph.,* **49**: 270-272. 1944.

Report of a case.—J. G. K.

Kaposi's Sarcoma and Lymphatic Leukemia. Report of a Case with Histologic Evidence of the Two Diseases in the Same Lesion. SACHS, W., and GRAY, M. [New York Post-Graduate Med. Sch. and Hosp., Columbia Univ., New York, N. Y.] *Arch Dermat. & Syph.,* **51**:325-329. 1945.

A case report.—J. G. K.

Bowen's Disease Associated with Anaplastic Carcinomatous Tumour. MITCHELL-HEGGS, G. B., and CROW, K. D., *Proc. Roy. Soc. Med.,* **39**:687. 1946.

Description of a case.—E. L. K.

Adenoma Sebaceum. With Report of a Case. ARONSTAM, N. E. [Detroit, Mich.] *M. Rec.* **157**:411-412. 1944.

In the cases of adenoma sebaceum which the author has observed, the disease was not (contrary to other reports) related to mental retardation, and was not alleviated by hormones, vitamins, physiotherapy, or any other measures tried. One case is presented in detail, in which no improvement followed 6 months of therapy by x-ray, ultraviolet radiation, foreign proteins, Fowler's solution, vitamin B complex, or endocrine preparations.—M. H. P.

NERVOUS SYSTEM

Notes on the Pathology of Cranial Tumors. 1. Osteomas of the Skull with Incidental Mention of Their Occurrence in the Ancient Incas. ABBOTT, K. H., and COURVILLE, C. B. [Coll. of Med. Evangelists, and Los Angeles County Hosp., Los Angeles, Calif.] *Bull. Los Angeles Neurol. Soc.,* **10**:19-34. 1945.

An illustrated review, with bibliography, and a description of osteomas found in Inca skulls at the San Diego Museum is given. In 3 of the Inca specimens, the frontal bone was the seat of the growth, in 2 the parietal bone, and in 2 the occipital bone.

The available facts regarding the location and development of osteomas of the skull suggest that these tumors result from some perversion in the union of the suture lines, possibly with the formation of isolated cell rests of osseous or cartilagenous character.—M. H. P.

Notes on the Pathology of Cranial Tumors. 2. Metastatic Tumors of the Calvarium with Incidental Reference to Their Occurrence in American Aborigines. COURVILLE, C. B., and ABBOTT, K. H. [Coll. of Med. Evangelists, and Los Angeles County Hosp., Los Angeles, Calif.] *Bull. Los Angeles Neurol. Soc.,* **10**:129-154. 1945.

Six instances of metastatic lesions of the skull were found in 30,000 autopsies at Los Angeles County Hospital; the primary lesions were in the breast, uterus, lung, and thigh. A seventh case is presented in which the cranial metastasis from a thyroid tumor was observed while the patient was still alive. Seven ancient American skulls studied in the San Diego Museum are described, in which defects, apparently arising from myelomas or metastatic tumors, appeared in the cranial vault. The literature on metastatic tumors of the skull is reviewed, with an extensive bibliography.—M. H. P.